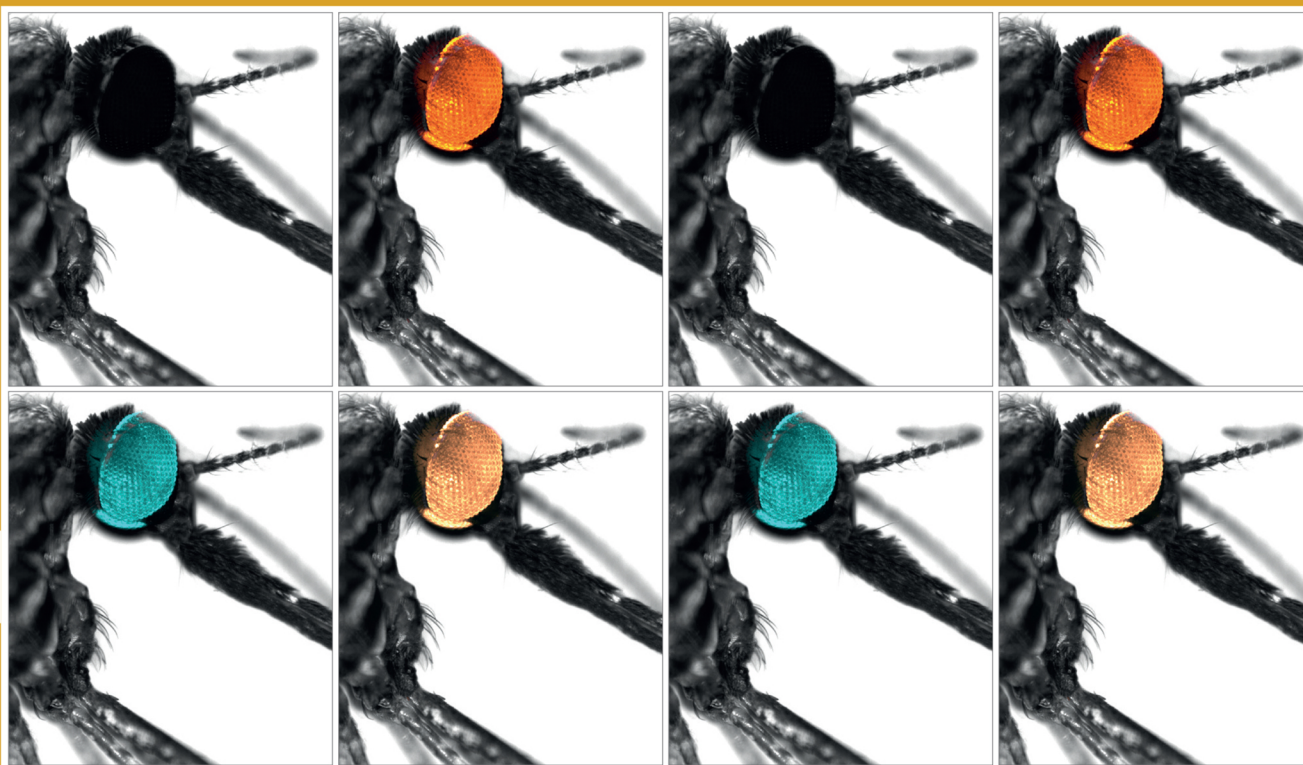**10**

Transgenic Insects 2nd Edition

Techniques and Applications

EDITED BY **MARK Q. BENEDICT** AND **MAXWELL J. SCOTT**

CABI

Transgenic Insects:

Techniques and Applications

_____

CABI Biotechnology Series

Biotechnology, in particular the use of transgenic organisms, has a wide range of applications including agriculture, forestry, food and health. There is evidence that it could make a major impact in producing plants and animals that are able to resist stresses and diseases, thereby increasing food security. There is also potential to produce pharmaceuticals in plants through biotechnology, and provide foods that are nutritionally enhanced. Genetically modified organisms can also be used in cleaning up pollution and contamination. However, the application of biotechnology has raised concerns about biosafety, and it is vital to ensure that genetically modified organisms do not pose new risks to the environment or health. To understand the full potential of biotechnology and the issues that relate to it, scientists need access to information that not only provides an overview of and background to the field, but also keeps them up to date with the latest research findings.

This series, which extends the scope of CABI's successful "Biotechnology in Agriculture" series, addresses all topics relating to biotechnology including transgenic organisms, molecular analysis techniques, molecular pharming, in vitro culture, public opinion, economics, development and biosafety. Aimed at researchers, upper-level students and policy makers, titles in the series provide international coverage of topics related to biotechnology, including both a synthesis of facts and discussions of future research perspectives and possible solutions.

Titles Available

1. Animal Nutrition with Transgenic Plants
   Edited by G. Flachowsky

2. Plant-derived Pharmaceuticals: Principles and Applications for Developing Countries
   Edited by K.L. Hefferon

3. Transgenic Insects: Techniques and Applications
   Edited by M.Q. Benedict

4. Bt Resistance: Characterization and Strategies for GM Crops Producing Bacillus thuringiensis Toxins
   Edited by Mario Soberón, Yulin Gao and Alejandra Bravo

5. Plant Gene Silencing: Mechanisms and Applications
   Edited by Tamas Dalmay

6. Ethical Tensions from New Technology: The Case of Agricultural Biotechnology
   Edited by Harvey James

7. GM Food Systems and their Economic Impact
   Tatjana Brankov and Koviljko Lovre

8. Endophyte Biotechnology: Potential for Agriculture and Pharmacology
   Edited by Alexander Schouten

9. Forest Genomics and Biotechnology
   Richard Meilan and Matias Kirst

10. Transgenic Insects: Techniques and Applications, 2nd Edition
    Edited by M.Q. Benedict and Maxwell J. Scott

Transgenic Insects:

Techniques and Applications

_____

Edited by

Mark Q. Benedict

Centers for Disease Control and Prevention (CDC)

and

Maxwell J. Scott

North Carolina State University

CABI

CABI is a trading name of CAB International

Contents

———————

3 Sex-, Tissue- and Stage-Specific Transgene Expression 42

Tony Nolan and Andrew M. Hammond

Contributors

—————————————

Hassan M.M. Ahmed, Department of Developmental Biology, Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology, GZMB, Georg-August-University Göttingen, Ernst-Caspari-Haus, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany. Email: hmutasi@biologie.uni-goettingen.de

Omar S. Akbari, Division of Biological Sciences, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093, USA. Email: oakbari@ucsd.edu

Serap Aksoy, Yale School of Public Health, Department of Epidemiology of Microbial Diseases, 60 College St. New Haven, CT 06520, USA. Email: serap.aksoy@yale.edu

Yehonatan Alcalay, Department of Entomology, Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot 7610001, Israel. Email: yehonatanal@mail.huji.ac.il

Luke Alphey, The Pirbright Institute, Ash Road, Woking, Surrey, GU24 0NF, UK. Email: luke.alphey@pirbright.ac.uk

Yael Arien, Department of Entomology, Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot 7610001, Israel. Email: yael.catz@mail.huji.ac.il

Rotem Daniel Avraham, Department of Entomology, Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot 7610001, Israel. Email: rotem.avraham@mail.huji.ac.il

Camilla Beech, Cambea Consulting Ltd, c/o 10 Beech Court, Reading, RG10 0RQ, UK. Email: cambeaconsults@gmail.com

Mark Q. Benedict, Centers for Disease Control and Prevention (CDC), Division of Parasitic Diseases and Malaria/Entomology Branch, 1600 Clifton Road NE, Atlanta, GA 30329, USA. Email: Mbenedict@cdc.gov

Nicholas J. Bongio, Duquesne University, Department of Biological Sciences, 600 Forbes Ave., Pittsburgh, PA 15282, USA. Email: nicholasjbongio@gmail.com

Vanessa Bottino-Rojas, Department of Microbiology and Molecular Genetics, University of California, 2315 McGaugh Hall, Irvine, CA 92627, USA. Email: bottinor@uci.edu

L. Roman Carrasco, Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, 117546, Singapore, Republic of Singapore. Email: dbsctlr@nus.edu.sg

Lucy Carter, CSIRO Land and Water, GPO Box 2583, Brisbane, QLD 4001, Australia. Email: Lucy.Carter@csiro.au

Jackson Champer, Center for Bioinformatics, School of Life Sciences, Peking University, Peking-Tsinghua Center for Life Sciences, Room 334-1, Wang Kezhen Building, Peking University, No. 5 Yiheyuan Road, Haidian District, Beijing, China 100871. Email: jchamper@pku.edu.cn

Isabelle Coche, EmergingAg, Suite 1320, 720 13th Avenue SW, Calgary, AB, T2R 1M5, Canada. Email: Isabelle@emergingag.com

Carolina Concha, Smithsonian Tropical Research Institute, Roosevelt Avenue, Tupper Building 401, Balboa, Ancon, Panama City, Panama. Email: carolacmcl@yahoo.com

Rocco D'Amato, Polo di Genomica, Genetica e Biologia, Via Mazzieri, 05100 Terni, Italy. Email: r.damato@pologgb.com

Brinda Dass, Foundation for the National Institutes of Health, 11400 Rockville Pike, Suite 600, North Bethesda, MD 20852, USA. Email: bdass@fnih.org

Jason A. Delborne, Department of Forestry and Environmental Resources, North Carolina State University, Campus Box 8008, Raleigh, NC 27695-8008, USA. Email: jadelbor@ncsu.edu

Ellen M. Dotson, Centers for Disease Control and Prevention (CDC), Division of Parasitic Diseases and Malaria/Entomology Branch, 1600 Clifton Rd NE, Atlanta, GA 30329, USA. Email: edotson@cdc.gov

Ravi V. Durvasula, Mayo Clinic Florida, 4500 San Pablo Road, Jacksonville, FL 32224, USA. Email: tuanravi@gmail.com

Matthew P. Edgington, The Pirbright Institute, Ash Road, Woking, Surrey, GU24 0NF, UK. Email: matt.edgington@pirbright.ac.uk

Claudia Emerson, Institute on Ethics & Policy for Innovation, L. R. Wilson Hall, 3011, McMaster University, 1280 Main Street West, Hamilton, Ontario, L8S 4K1, Canada. Email: emersoc@mcmaster.ca

Guido Favia, School of Biosciences and Veterinary Medicine, Via Gentile III da Varano, 62032 Camerino (MC), Italy. Email: guido.favia@unicam.it

Adam P. Forshaw, Department of Anesthesiology, University of Wisconsin, Madison, 600 Highland Ave., Madison, WI 53792, USA. Email: apforshaw@gmail.com

Alexander W.E. Franz, Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri, 303 Connaway Hall, Columbia, MO 65211, USA. Email: franza@missouri.edu

Marisa L. Guido, Duquesne University, Department of Biological Sciences, 600 Forbes Ave., Pittsburgh, PA 15282, USA. Email: guidom@duq.edu

Monika Gulia-Nuss, Department of Biochemistry and Molecular Biology, University of Nevada, Reno, NV 89557, USA. Email: mgulianuss@unr.edu

Daniella An Haber, Department of Entomology, Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot 7610001, Israel. Email: daniella.haber@mail.huji.ac.il

Andrew Hammond, Johns Hopkins Malaria Research Institute, Department of Molecular Microbiology & Immunology, Johns Hopkins Bloomberg School of Public Health, 615 N. Wolfe Street, W4104-3, Baltimore, MD 21205, USA. Email: ahammo20@jhmi.edu

Alfred M. Handler, USDA-ARS, CMAVE, 1600 SW 23rd Dr., Gainesville, FL 32608, USA. Email: ahandler@ufl.edu

Keith R. Hayes, CSIRO, Data61, Castray Esplanade, Hobart, Tasmania, Australia. Email: keith.hayes@data61.csiro.au

Ivy Hurwitz, Center for Global Health, University of New Mexico Health Sciences Center, 915 Camino de Salud, IDTC 3150, Albuquerque, NM 87131, USA. Email: IHurwitz@salud.unm.edu

Anthony A. James, Departments of Microbiology & Molecular Genetics and Molecular Biology & Biochemistry, 3205 McGaugh Hall, University of California, Irvine, CA 92697, USA. Email: aajames@uci.edu

Thomas P. Kelly, Duquesne University, Department of Biological Sciences, 600 Forbes Ave., Pittsburgh, PA 15282, USA. Email: kellyt3@duq.edu

Nicole Klein, Sawtooth Epidemiology and Infectious Disease, 125 East Idaho St, Suite 100, Boise, ID 83712, USA. Email: nic72karin@gmail.com

Ana Kormos, 782 Apricot Avenue, Winters, CA 95694, USA. Email: amkormos@gmail.com

Grzegorz Krawczyk, Department of Entomology, Room W127, Millennium Science Complex, The Pennsylvania State University, University Park, PA 16802, USA. Email: gxk13@psu.edu

Lee Benjamin Lamdan, Department of Entomology, Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot 7610001, Israel. Email: lee.lamdan@mail.huji.ac.il

David J. Lampe, Duquesne University, Department of Biological Sciences, 600 Forbes Ave., Pittsburgh, PA 15282, USA. Email: lampe@duq.edu

Rosemary S. Lees, Department of Vector Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA, UK. Email: rosemary.lees@lstmed.ac.uk

Judy Li, Department of Biochemistry and Molecular Biology, Michael Smith Laboratories, University of British Columbia, 436–2125 East Mall, Vancouver, BC V6T1K4, Canada. Email: judysiqi@gmail.com

Alison McAfee, Department of Biochemistry and Molecular Biology, Michael Smith Laboratories, University of British Columbia, 436–2125 East Mall, Vancouver, BC V6T1K4, Canada. alison.n.mcafee@gmail.com

Vanessa M Macias, Department of Entomology, Room W127, Millennium Science Complex, The Pennsylvania State University, University Park, PA 16802, USA. Email: Vanessa.Macias@unt.edu

Maria Vittoria Mancini, Polo di Genomica, Genetica e Biologia, Via Mazzieri, 05100 Terni, Italy. Email: mv.mancini@pologgb.com

Aditi Mankad, CSIRO Land and Water, GPO Box 2583, Brisbane, QLD 4001, Australia. Email: aditi.mankad@csiro.au

Sage McKeand, Department of Entomology, Room W127, Millennium Science Complex, The Pennsylvania State University, University Park, PA 16802, USA. Email: sgm5201@psu.edu

Neil I. Morrison, Oxitec Limited, 71 Milton Park, Oxford, OX14 4RQ United Kingdom, Email: Neil.Morrison@oxitec.com

John D. Mumford, Centre for Environmental Policy, Imperial College London, Silwood Park Campus, Ascot, Berkshire, SL5 7PY, UK. Email: j.mumford@imperial.ac.uk

Tony Nolan, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA, UK. Email: Tony.Nolan@lstmed.ac.uk

Andrew Nuss, Agriculture, Veterinary, and Rangeland Sciences Department, University of Nevada, Reno, NV 89557, USA. Email: nussab@unr.edu

David A. O'Brochta, Department of Entomology, University of Maryland, College Park. Present address: 11400 Rockville Pike Suite 600, North Bethesda, MD 20852, USA. Email: dobrochta@fnih.org

Marianne Otte, Department of Evolutionary Genetics, Heinrich Heine University Düsseldorf, 26.14 Universitätsstraße 1, Düsseldorf 40225, Germany. Email: marianne.otte@email.de

Riccardo Papa, University of Puerto Rico - Río Piedras, Julio Garzía Díaz (JGD) 213 Rio Piedras, Avenida Gandara, Rio Piedras, San Juan, PR 0093, Puerto Rico. Email: rpapa.lab@gmail.com

Philippos Aris Papathanos, Department of Entomology, Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot 7610001, Israel. Email: p.papathanos@mail.huji.ac.il

Ricardo Pereira, Cardiff University, Law & Politics School, Cardiff, CF10 3AX, UK. Email: PereiraR1@cardiff.ac.uk

Hillary Peterson, Department of Entomology, Room W127, Millennium Science Complex, The Pennsylvania State University, University Park, PA 16802, USA. Email: hillary.m.peterson@gmail.com

Michael Pham, Department of Biochemistry and Molecular Biology, University of Nevada, Reno, NV 89557, USA. Email: mpham@unr.edu

Megan Quinlan, Centre for Environmental Policy, Imperial College London, Silwood Park Campus, Ascot, Berkshire, SL5 7PY, UK. Email: m.quinlan@imperial.ac.uk

Robyn Raban, Division of Biological Sciences, Section of Cell and Developmental Biology, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093, USA. Email: rraban@ucsd.edu

Jason L. Rasgon, Department of Entomology, Room W127, Millennium Science Complex, The Pennsylvania State University, University Park, PA 16802, USA. Email: jlr54@psu.edu

Aaron Roberts, Institute on Ethics & Policy for Innovation, L.R. Wilson Hall, 3011, McMaster University, 1280 Main Street West, Hamilton, Ontario, L8S 4K1, Canada. Email: robera21@mcmaster.ca

Yasha Rohwer, Department of Humanities and Social Sciences, Oregon Institute of Technology, 3201 Campus Drive, Klamath Falls, OR 97601, USA. Email: yrohwer@gmail.com

Nathan Rose, Oxitec Ltd, 71 Innovation Drive, Milton Park, Abingdon, OX14 4RQ, UK. Email: nathan.rose@oxitec.com

Marc F. Schetelig, JLU Giessen, Department of Insect Biotechnology in Plant Protection, Winchesterstrasse. 2, 35394 Giessen, Germany. Email: marc.schetelig@agrar.uni-giessen.de

Kristof De Schutter, Laboratory of Agrozoology, Department of Plants and Crops, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium. Email: Kristof.DeSchutter@UGent.be

Maxwell J. Scott, Department of Entomology and Plant Pathology, North Carolina State University, Campus Box 7613, Raleigh, NC 27695-7613, USA. Email: mjscott3@ncsu.edu

Hideki Sezutsu, Division of Silk-producing Insects Research, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization (NARO), 1-2 Owashi, Tsukuba, Ibaraki, 305-8634 Japan. Email: hsezutsu@affrc.go.jp

Arvind Sharma, Department of Biochemistry and Molecular Biology, University of Nevada, Reno, NV 89557, USA. Email: arvindsharma.phd@gmail.com

Gregory S. Simmons, USDA/APHIS/PPQ, Science and Technology, 1636 East Alisal Street Salinas, California, 93905, USA. Email: gregory.s.simmons@usda.gov

Guy Smagghe, Laboratory of Agrozoology, Department of Plants and Crops, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium. Email: Guy.Smagghe@UGent.be

Toshiki Tamura, Silk Sciences and Technology Research Institute, 1053 Iigura, Ami-machi, Inashiki-gun, Ibaraki, 300-0324 Japan. E-mail: monsan20@yahoo.co.jp

Gerard Terradas, Department of Entomology, Room W-242, Millennium Science Complex, The Pennsylvania State University, University Park, PA 16802, USA. Email: gkt5113@psu.edu

Delphine Thizy, rue Alphone Hottat 35, 1050 Ixelles, Belgium. Email: delphine@thizyconsulting.com

Lea Pare Toe, Institut de Recherche en Sciences de la Santé – IRSS, BP 545, Bobo Dioulasso, Burkina Faso. Email: lea_toe@yahoo.com

Brian L. Weiss, Yale School of Public Health, Department of Epidemiology of Microbial Diseases, 60 College St. New Haven, CT 06520, USA. Email: brian.weiss@yale.edu

Ernst A. Wimmer, Department of Developmental Biology, Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology, GZMB, Georg-August-University Göttingen, Ernst-Caspari-Haus, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany. Email: ewimmer@gwdg.de

Liu Yang, Yale School of Public Health, Department of Epidemiology of Microbial Diseases, 60 College St. New Haven, CT 06520, USA. Email: liu.yang.ly274@yale.edu

Preface to the 2nd Edition

There have been a few pivotal technology advances that have facilitated the development of technologies for genetic engineering of insects; for example, the identification of transposons such as *piggyBac* and *Minos* that have a wide host range; and the development of fluorescent protein marker genes. More often, improvements in germline transformation efficiency are due to small incremental steps such as changes in needle design and embryo handling.

Since the publication of the first edition of this volume, there has been one game-changing technology, CRISPR/Cas, which has revolutionized genetic manipulation of insect genomes. Few chapters in this book do not mention the use of CRISP/Cas, which is used in many disparate ways, from creating knock-ins and knockouts to gene drives. The rapid development in the latter has led us to include several new chapters in this edition, on gene editing and gene drive technology. The chapters on the considerations for release of transgenic insects have been updated to consider gene drive in addition to reviewing the experience gained from several open field trials that have been performed over the past few years.

Germline transformation and gene editing of some insects remains a major challenge. Delivery of DNA to create modifications has also advanced in the form of Receptor-Mediated Ovary Transduction of Cargo, known as ReMOT Control. Particularly for species with embryos that are difficult to microinject, this allows a simple method for gene editing. In addition to a chapter on CRISPR/Cas9 gene editing, we have included a chapter on RNAi as this remains a very useful technology for targeted control of gene expression.

While there is much excitement around gene drive technology, researchers have continued to develop, improve and test insects carrying tetracycline-repressible female-lethal genes, which is also known as fs-RIDL®. Consequently, the chapters on fs-RIDL® have been retained and updated. Similarly, the chapters on paratransgenic control of insects have been updated as this remains a promising approach, particularly for species that are difficult to transform, such as tsetse fly.

We have also included a chapter on a non-insect: ticks. While germline transformation has not been achieved, many of the tools that are described elsewhere are relevant for ticks. Furthermore, their human and animal health importance make the content a good fit for those interested in this volume.

We hope that readers will appreciate that transgenic technology sometimes inches forward but that many of the chapters in this book reflect those less-frequent leaps. For those readers at the early stages of their careers, the wide availability of reference genomes and the refinement of germline transformation and gene editing technologies make this a wonderful time for basic and applied research on insect genetics.

Preface to the 1st Edition

The advent of transgenic modification of model eukaryotes, particularly *Drosophila melanogaster*, stimulated expansion into species that are of particular intrinsic scientific interest, but more so into those that have industrial potential or whose pestiferousness might be mitigated by novel genetic approaches. Not surprisingly, species that are of high importance to public health and agriculture occupy prominent places. The ostensible *raison d'être* of such explorations is invariably developing an insect for a control or industrial application, though healthy scientific curiosity surely plays a role. Many of the molecular tools developed in model organisms have now been transferred to such non-model insects. As the reader will appreciate after reading even a few chapters of this volume, such transitions are often not made seamlessly but require specific modifications to obtain satisfactory results in the species of interest.

In the early days of *Drosophila* transgenesis when 'Rubin and Spradling' and 'Spradling and Rubin' were on every aspiring insect molecular biologist's tongue, I was awed by the seemingly unlimited potential that the diverse gene-regulation systems and effector combinations offered to modify insect phenotypes. The permutations seemed more than sufficiently numerous that any imaginable phenotype could be devised and engineered. While that expectation was naïve, some of the potential has in fact been realized, but not without considerable effort.

Delivering control and industry-ready transgenic insects especially is a difficult and often-perplexing enterprise. Great progress has been made in silkworm transgenesis, owing in part to the large number of strains that are maintained and the commercial potential for producing male-only strains and strains producing modified silk. Tephritid fruit flies have also enjoyed remarkable success in transgenic applications owing in part to the control applications that could clearly result, the ease of transformation compared to many other non-model species and the existence of genetic control programmes that are a natural entrée for transgenic improvements. The prior existence of non-transgenic sterile insect programmes has also been a factor that opens the door for rapid adoption of transgenic screwworm, blowflies and pink bollworm.

In my own field, mosquito biology, considerable transgenic insect work has resulted in only one transgenic mosquito that I consider a 'complete package' that has been released in the field for control purposes. Even that was made possible only by an intrinsic feature of *Aedes aegpti* that allows one to eliminate females mechanically by size rather than using transgenic technology. It is likely that if this technology, RIDL®, were applied to a major

malaria vector, *Anopheles gambiae*, that the inability to separate sexes *en masse* would mean a dead end for the application until some capacity to do so were developed. The versatility of the technology should not be underestimated however: the same technology has been adapted to several agricultural pests.

Some barriers to implementation of transgenic approaches to reduce pestiferousness are due to lack of knowledge and may be eliminated progressively by further research in genetics and molecular biology. The mechanisms of pathogen resistance, sex determination and gene modulation are all insufficiently understood in species of interest that they restrain transgenic applications. With time, research will reduce many of these barriers.

Other limitations are less amenable to a molecular biology solution. Large numbers of insect colonies of some species are simply difficult to maintain. Some require development of special skills to inject DNA into embryos without killing them. To reduce such hurdles, supporting technologies in manipulation, stock-keeping and embryo cryopreservation must advance before the full potential is realised. Tsetse flies develop a single larva at a time *in utero*, making creating transgenic insects problematic. Paratransgenesis avoids this and other challenges by utilizing the wealth of molecular tools available for bacteria and circumventing the need to create transgenic insects at all.

The creativity of scientists has and will result in transgenic and paratransgenic insect 'products' that – if they ever leave the laboratory – will face the more poorly defined requirements of acceptability, economy and regulatory compliance. The international mosaic of attitudes, agreements and applications makes this difficult territory for scientists who are accustomed to thinking of the next grant or manuscript and are not trained to navigate these shifting and confusing channels.

Even a perusal of this volume will impress upon the reader that the remarkable advances in insect transgenesis offer yet-undeveloped exciting prospects, frustrating difficulties and complex social implications. It is my hope that the contents will present not only technical information but will provide some familiarity with the breadth of issues that insect transgenesis involves. In my experience, scientists who are developing this technology wish to see its benefits realized in a safe and transparent way for the improvement of human welfare and environmental quality. To the extent that this collection accomplishes this, it has achieved its objectives.

Mark Q. Benedict

Acknowledgements

————————————

1 Transposon-Based Technologies for Insects

David A. O'Brochta*

Department of Entomology, University of Maryland, College Park

_____

1.1 Introduction

Insect science has benefited greatly from the variety of technologies created from transposons since their first use to develop germline transformation technology in the early 1980s (Rubin and Spradling, 1982; Spradling and Rubin, 1982). They have not only enabled the deep exploration of basic insect biology but have also enabled and continue to enable the development of new technologies for controlling and eliminating unwanted populations of insects of agricultural and public health importance (Gorman *et al*., 2016; Asadi *et al*., 2020; Shelton *et al*., 2020). After briefly reviewing the transposons currently known to be serviceable gene vectors in insects the range of applications of transposon-based vector systems in insects is considered, illustrating that cut-and-paste DNA-type transposons continue to have unique and important roles within an ever-expanding genome manipulation technology ecosystem. New advances in these technologies are reviewed, including modifications affecting the performance characteristics of the element-specific transposases such that their activity and precision are increased. Finally, the recent discovery of novel transposons that have been shown to be capable of serving as programmable integrases, combining the best attributes of modern gene editing technologies and transposons, could further increase the transposon technology options available to insect scientists interested in manipulating insect genotypes and phenotypes.

1.2 Transposons Used in Insects

The current complement of transposable elements with demonstrated functionality in insects is of a type known as cut-and-paste DNA transposons (Wicker *et al*., 2007; Kapitonov and Jurka, 2008; Muñoz-López and García-Pérez, 2010). DNA transposons that include the cut-and-paste type, *Helitrons* and *Polintons/Mavericks*, move within genomes using DNA intermediates. Cut-and-paste DNA transposons whose movement involves element excision and integration serve as versatile platforms for genome manipulation technologies in plants and animals, including humans and insects (Wicker *et al*., 2007; Muñoz-López and García-Pérez, 2010; Thomas *et al*., 2015; Kebriaei *et al*., 2017; Laptev *et al*., 2017; Amberger and

_____

*Email: dobrochta@fnih.org

Ivics, 2020) (Fig. 1.1). Based on current understanding of the biology, biochemistry and genetics of cut-and-paste DNA transposons it is likely that many, if not most, would function in insect cells. The abundance of these elements in genomes throughout the tree of life is a reflection, in part, of a general absence of host specificity and this in turn reflects their structural and biochemical simplicity. Cut-and-paste DNA transposons are structurally simple genetic elements whose mobility can be conferred essentially to any DNA sequence by attaching essential transposable element structural sequences (inverted terminal repeat sequences) and whose mobility can be controlled by regulating the expression of an element-specific transposase *in trans* (Muñoz-López and

García-Pérez, 2010) (Fig. 1.2). Consequently, they have become robust and popular platforms upon which a variety of genetic technologies have been assembled and deployed (Bouuaert and Chalmers, 2010; DeNicola *et al*., 2015; Amberger and Ivics, 2020; Cain *et al*., 2020; Fedorova and Dorogova, 2020; Kumar *et al*., 2020).

1.2.1   *P* elements

P elements were the first transposable elements to be isolated from insects and were originally discovered in *Drosophila melanogaster* as the genetic factors found in some wild-caught lines that induced a genetic syndrome

P element	87 kD	31 bp 8 bp
piggyBac	68 kD	13 bp TTAA
Minos	40 kD	255 bp TA
Hermes/hAT	70 kD	17 bp nTnnnn An
mariner/Mos1	41 kD	28 bp TA
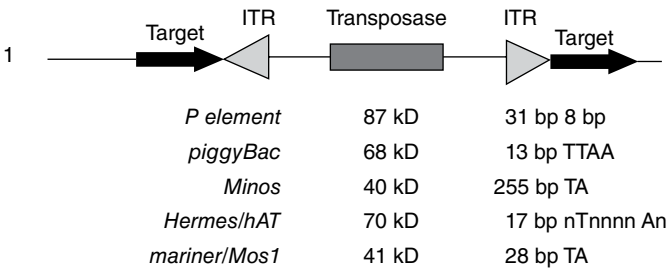
Fig. 1.1. Basic organization structure of cut-and-paste DNA transposon. Target: sequence motif into which the transposon integrates and is duplicated during the integration reaction; ITR: inverted terminal repeats; Transposase: a gene encoding the element-specific integrase.
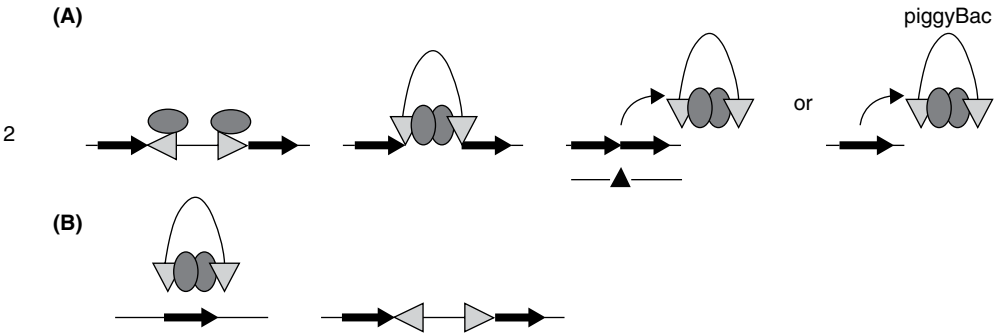
Fig. 1.2. (A) A general representation of a cut-and-paste DNA transposon excising from a chromosome. With the exception of *piggyBac* which excises precisely, restoring the integration site to its exact pre-integration state, cut-and-paste DNA transposons excise imprecisely, resulting in the addition or deletion of DNA at the integration site. **(B) Integration reaction resulting in the direct duplication of the element-specific target site.** Filled arrow: target site; filled ovals: transposase protein; filled triangle: inverted terminal repeat sequence

known as hybrid dysgenesis (Kidwell *et al.*, 1977). The high levels of transpositional activity of *P* elements in *D. melanogaster* have made them particularly useful as platforms for constructing genetic technologies in that species (Ryder and Russell, 2003). The *P* element has a loosely constrained 8 bp target sequence and in *D. melanogaster* it prefers to integrate in the 5′ regions of genes (Majumdar and Rio, 2015). With one exception, its activity has not been reported in insect species outside the family Drosophilidae (O'Brochta and Handler, 1988; Kim *et al.*, 2003) and this extremely limited host range is uncommon among cut-and-paste DNA transposons isolated from insects (Table 1.1).

1.2.2   *piggyBac*

The *piggyBac* transposon was discovered as an insertion sequence in a baculovirus gene that was shown to be of insect origin and probably arose during the passage of the virus through *Trichoplusia ni* cells *in vivo* (Fraser *et al.*, 1983; Fraser *et al.*, 1985). Native intact *piggyBac* elements are small: 2472 bp in length with 31 bp perfect terminal inverted repeats and a single 2.1 kb open reading frame encoding for a 64 kD transposase protein (Wang *et al.*, 1989) and, with rare exceptions, integrate into TTAA sites (Wang and Fraser, 1993; Bouallègue *et al.*, 2017). Like *P* and other elements, *piggyBac*'s pattern of integration in insect genomes is non-random although in *D. melanogaster* this pattern is quite distinct for that observed with *P* and other elements (Thibault *et al.*, 2004; Bellen *et al.*, 2011). The well-documented broad host range of *piggyBac* which spans eukaryotes makes it the most popular insect gene vector and 45 species of insect have been reported to have been transformed using *piggyBac* gene vectors (Yusa, 2015) (Table 1.1). While the activities of all cut-and-paste DNA transposon gene vectors appear to be inversely correlated with the amount of transgenic DNA the elements are carrying, *piggyBac* has been reported to be active with 100 kb of transgene DNA (Li *et al.*, 2011). This element is unusual in that

excision perfectly restores the integration site to its pre-integration state ('scarless excision'), something not seen with any other element currently used as a gene vector (Fig. 1.2). These elements are widely distributed within insect species and many closely resemble the element used as a vector, suggesting that horizontal transfer has occurred (Zimowska and Handler, 2006). That endogenous *piggyBac* elements might interfere with the effective use of the popular *piggyBac* gene vector is worth considering, as cross-mobilization of related transposons has been reported (Sundararajan *et al.*, 1999). While *piggyBac* shows almost invariable preference for integrating into TTAA target sites, *piggyBac*'s pattern of integration in insect genomes is non-random, although in *D. melanogaster* this pattern is quite distinct for that observed with *P* and other elements (Thibault *et al.*, 2004; Bellen *et al.*, 2011). Hyperactive variants of *piggyBac* transposase have been developed in non-insect systems and some also appear to have elevated levels of activity in insects (Yusa *et al.*, 2011; Eckermann *et al.*, 2018; Wen *et al.*, 2020).

1.2.3   *Mos1*

Mos1 is a *Tc1/mariner* element from *Drosophila mauritiana* (Bryan *et al.*, 1987; Medhora *et al.*, 1988) that has been successfully used as a gene vector in only *D. melanogaster*, *D. virilis*, *Aedes aegypti* and *Tribolium castaneum* (Table 1.1). The *Tc1/mariner* superfamily of transposons is perhaps the most widespread group of transposons, present throughout the tree of life (Tellier *et al.*, 2015). The rates of transposition of *mariner* in these species are low relative to other gene vectors and this has discouraged the widespread use of *Mos1* in insects. Transposase variants have been created that have elevated activity and some have elevated activities in insects (Pledger and Coates, 2005; Liu and Chalmers, 2014). However, while of limited utility in insects, *Tc1/mariner* elements from insects have proven to be highly active in bacteria and

Table 1.1. Transposons used for insect germline transformation.

Type	Element	Order	Genus	Species	Reference
Composite					
	Tn5	Diptera	*Aedes*	*aegypti*	Rowan *et al.*, 2004
P					
	P	Diptera	*Drosophila*	*melanogaster*	Spradling and Rubin, 1982
				hawaiiensis	Brennan *et al.*, 1984
				simulans	Scavarda and Hartl, 1984
		Lepidoptera	*Bombyx*	*mori*	Kim *et al.*, 2003
hAT					
	Hermes	Coleoptera	*Tribolium*	*castaneum*	Berghammer *et al.*, 1999
		Diptera	*Aedes*	*aegypti*	Jasinskiene *et al.*, 1998
			Culex	*quinquefasciatus*	Allen *et al.*, 2001
			Ceratitis	*capitata*	Michel *et al.*, 2001
			Drosophila	*melanogaster*	O'Brochta *et al.*, 1996
			Stomoxys	*calcitrans*	O'Brochta *et al.*, 2000
		Lepidoptera	*Bicyclus*	*anynana*	Marcus *et al.*, 2004
	Herves	Diptera	*Drosophila*	*melanogaster*	Arensburger *et al.*, 2005
	hobo	Diptera	*Drosophila*	*melanogaster*	Blackman and Gelbart, 1989
				virilis	Lozovskaya *et al.*, 1996
		Lepidoptera	*Helicoverpa*	*zea*	DeVault *et al.*, 1996
	hopper	Diptera	*Anastrepha*	*suspensa*	Handler and O'Brochta, 2012
			Bactrocera	*dorsalis*	Handler and Schetelig, 2020
			Drosophila	*melanogaster*	Handler and O'Brochta, 2012
mariner/Tc1					
	mariner	Coleoptera	*Tribolium*	*castaneum*	Berghammer *et al.*, 1999
		Diptera	*Aedes*	*aegypti*	Coates *et al.*, 1998
			Drosophila	*melanogaster*	Garza *et al.*, 1991
				virilis	Lohe and Hartl, 1996
	Minos	Coleoptera	*Tribolium*	*castaneum*	Pavlopoulos *et al.*, 2004
			Hypothenemus	*hampei*	O'Brochta, unpublished
			Diabrotica	*virgifera virgiferai*	Chu *et al.*, 2017
		Diptera	*Anopheles*	*stephensi*	Catteruccia *et al.*, 2000
			Bactrocera	*oleae*	Koukidou *et al.*, 2006
			Ceratitis	*capitata*	Loukeris *et al.*, 1995b
			Teleopsis	*dalmanni*	Warren *et al.*, 2010
			Drosophila	*melanogaster*	Loukeris *et al.*, 1995a
				suzukii	Chu *et al.*, 2018
		Lepidoptera	*Bombyx*	*mori*	Uchino *et al.*, 2008
TTAA-specific					
	piggyBac	Orthoptera	*Gryllus*	*bimaculatus*	Nakamura *et al.*, 2010
		Coleoptera	*Harmonia*	*axyridis*	Kuwayama *et al.*, 2006
			Tribolium	*castaneum*	Berghammer *et al.*, 1999
			Hypothenemus	*hampei*	O'Brochta, unpublished
		Diptera	*Aedes*	*aegypti*	Kokoza *et al.*, 2001
				albopictus	Labbé *et al.*, 2010
				fluviatilis	Rodrigues *et al.*, 2006
			Anopheles	*albimanus*	Perera *et al.*, 2002
				arabiensis	Bossin *et al.*, 2006

Continued

Table 1.1. Continued.

Type	Element	Order	Genus	Species	Reference
				gambiae	Grossman et al., 2001
				sinensis	Liu et al., 2021
				stephensi	Nolan et al., 2002
			Anastrepha	ludens	Condon et al., 2007
				suspensa	Handler and Harrell, 2001
			Bactrocera	dorsalis	Handler and McCombs, 2000
				oleae	Genc et al., 2016
				tryoni	Raphael et al., 2011
			Ceratitis	capitata	Handler et al., 1998
			Chironomus	riparius	Caroti et al., 2015
			Cochliomyia	hominivorax	Allen et al., 2004
			Drosophila	ananassae	Holtzman et al., 2010
				erecta	Holtzman et al., 2010
				mauritiana	Stern et al., 2017
				melanogaster	Handler and Harrell, 1999
				mojavensis	Holtzman et al., 2010
				pseudoobscura	Holtzman et al., 2010
				santomea	Stern et al., 2017
				sechellia	Holtzman et al., 2010
				simulans	Holtzman et al., 2010
				suzukii	Schetelig and Handler, 2013
				virilis	Holtzman et al., 2010
				willistoni	Finokiet et al., 2007
				yakuba	Holtzman et al., 2010
			Lucilia	cuprina	Heinrich et al., 2002
				sericata	Concha et al., 2011
			Megaselia	abdita	Caroti et al., 2015
			Musca	domestica	Hediger et al., 2001
			Teleopsis	dalmanni	Warren et al., 2010
		Lepidoptera	Bicyclus	anynana	Marcus et al., 2004
			Bombyx	mori	Tamura et al., 2000
			Cydia	pomonella	Ferguson et al., 2011
			Junonia	coenia	Bossin et al., 2007
			Ostrinia	furnacalis	Liu et al., 2012
			Pectinophora	gossypiella	Peloquin et al., 2000
			Plutella	xylostella	Martins et al., 2012
		Hymenoptera	Apis	mellifera	Schulte et al., 2014
			Athalia	rosae	Sumitani et al., 2003

are used extensively as functional genomics tools in these organisms (Picardeau, 2010).

1.2.4 *Minos*

Minos is a *mariner*-like element from *Drosophila hydei* that is only 1.4 kb in length and is somewhat different from the other elements used as insect gene vectors in that the inverted terminal repeats are relatively long at 255 bp, and the transposase gene consists of two exons separated by a small 60 bp intron (Franz and Savakis, 1991; Franz et al., 1994) (Fig. 1.1). Like other *Tc1/mariner* elements, *Minos* integrates into TA dinucleotide target sites. Within insects, *Minos* has

been used to create transgenic *D. melanogaster, D. suzukii, Ceratitis capitata, Bactrocera oleae, Anopheles stephensi, Bombyx mori, T. castaneum* and *Teleopsis dalmanni* (Table 1.1). In *D. melanogaster*, the pattern of *Minos* integrations closely approximated a random distribution when considering distributions along each chromosome. *P* and *piggyBac* elements under the same conditions showed distinctly non-random distributions (Bellen *et al.*, 2011). The mobility properties of *Minos* in *D. melanogaster* and the differences from *P* and *piggyBac* have made *Minos* a very valuable asset in efforts to mutagenize all protein-coding genes of this species. *Minos* has a broad host range extending beyond insects to include amphipods, ascidians and mammals (Drabek *et al.*, 2003; Sasakura *et al.*, 2003; Pavlopoulos and Averof, 2005).

1.2.5 *Hermes*, *Herves*, *hopper* and *hobo*

The *hAT* family of transposons is widely distributed throughout the plant and animal kingdoms and they have been used effectively as insect germline transformation vectors in a range of species (Kempken and Windhofer, 2001; Arensburger *et al.*, 2011) (Fig. 1.1). *Hermes* is an active *hAT* transposon with a broad host range in insect and non-insect species isolated originally from *Musca domestica* (Atkinson *et al.*, 1993; Warren *et al.*, 1994). It has been used as an insect germline transformation vector in *D. melanogaster, C. capitata, Ae. aegypti, Culex quinquefasciatus, T. castaneum* and the butterfly *Bicyclus anynana* and has transposition/integration rates comparable to other commonly used transposons (Guimond *et al.*, 2003) (Table 1.1). While *Herves* from *Anopheles gambiae* (Arensburger *et al.*, 2005), *hopper* from *Bactrocera dorsalis* (Handler and Schetelig, 2020) and *hobo* from *D. melanogaster* (McGinnis *et al.*, 1983) have also been used effectively as gene vectors in insects, less is known about their host ranges and their patterns of integration (Smith *et al.*, 1993) (Table 1.1). However, they are not expected to differ greatly from *Hermes*.

1.2.6 *Tn5*

Tn5 is a composite transposon originally isolated from bacteria consisting of two copies of the insertion sequence IS50 flanking three antibiotic resistance genes (Hayes, 2003; Reznikoff, 2008). *Tn5* has been well characterized at the biochemical level and developed into a versatile functional genomics tool primarily for prokaryotes, although it has been shown to be an effective gene vector in an insect and other eukaryotes as well as a probe for genomics research (Goryshin *et al.*, 2000; Shi *et al.*, 2002; Suganuma *et al.*, 2005; Rowan *et al.*, 2004; Li *et al.*, 2020). A unique aspect of this system that distinguishes it from other insect vector systems is the commercial availability of purified hyperactive *Tn5* transposase, enabling the introduction of preassembled *Tn5* transpososomes (Kirby, 2007).

1.3 Mutagenesis

In their simplest form, active transposons can be powerful mutagens whose integration into promoters, exons, introns, and 3′ and 5′ regions of genes can alter and disrupt gene expression. These integration and mutagenic properties can be used to identify and subsequently isolate genes and have been used with great effect in plants, animals and insects (Kawakami *et al.*, 2017; Shelake *et al.*, 2019). The mutagenic potential of highly active transposons in *D. melanogaster* has been the basis for identifying and mutating every protein-coding gene in *D. melanogaster*. While these efforts initially relied solely on the mutagenic properties of *P* elements (Bellen *et al.*, 2004, 2011), because cut-and-paste DNA transposons have non-random and dissimilar integration site preferences a single transposon such as the *P* element would not be able to reach all genes. As other active transposon systems were discovered in, and isolated from, other insects, they too were used as mutagens in *D. melanogaster*, resulting in more complete coverage of the genome with transposon mutations (Thibault *et al.*, 2004; Metaxakis

et al., 2005). While the transposons used to mutagenize the genome of *D. melanogaster* were initially small non-autonomous elements with little more than a genetic marker, as other gene manipulation technologies emerged mutator elements were modified to contain other sequences that enhanced the potential of those elements to be used for other post-integration modifications of the genome as described briefly below (Bellen *et al.*, 2011; Nagarkar-Jaiswal *et al.*, 2015a; Nagarkar-Jaiswal *et al.*, 2015b; Lee *et al.*, 2018). Using similar strategies but on a smaller scale, similar transgenic resources have been created for *Drosophila simulans*, *D. mauritiana*, *D. yakuba*, *D. santomea* and *D. virilis* (Stern *et al.*, 2017).

Transposon-based mutagenesis has also been conducted on a large scale in the red flour beetle, *T. castaneum*. In this case a *piggyBac* transposon was introduced into the genome of *T. castaneum* and then induced to transpose at high rates. Trauner *et al.* (2009) generated 6816 *piggyBac* insertions in their study; of the 5657 insertion lines tested for lethality and sterility, 421 (7.4 %) were confirmed as homozygous lethal lines and eight (0.1 %) were homozygous sterile (Trauner *et al.*, 2009). While Trauner *et al.* (2009) demonstrated the feasibility of using transposons as forward genetic tools in non-drosophilid insects, the adoption of this technology is constrained by practical considerations such as one's ability to maintain and preserve the valuable genetic resources in the form of distinct genetic lines that emerges from such studies (Trauner *et al.*, 2009).

1.4 Germline Transformation

The potential of transposons to be used as gene vectors in insects for the purposes of introducing new DNA sequences into genomes was realized shortly after the initial isolation of the *P* element from *D. melanogaster* (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Today there are multiple transposon-based gene vectors available to insect scientists. The mobility properties of DNA transposons and their structural simplicity make them particularly amenable to being modified to carry genes and other DNA sequences into genomes. Attaching transposon sequences that serve a structural role in the transposition process, the inverted terminal repeat sequences, to any sequence of DNA confers upon that DNA many of the mobility properties of the original transposon in the presence of transposon-specific transposase protein (Fig. 1.3). Using transposons as gene vectors to move genes and other DNA sequences into genomes is one of the most common transposon-based technologies and is the foundation upon which a number of other genetic technologies have been created (Ivics and Izsvak, 2010). Because the organization of DNA
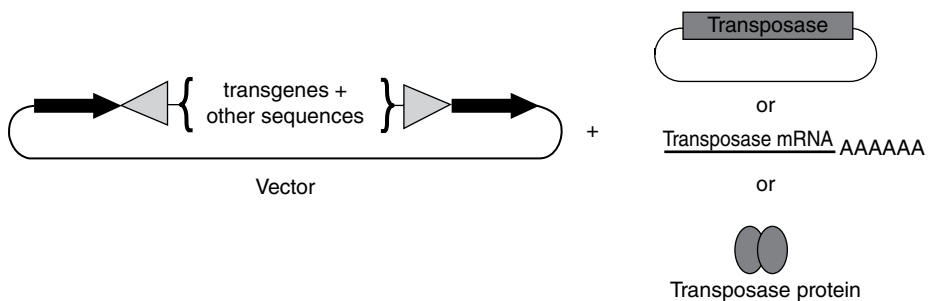
Fig. 1.3. General representation of binary vector systems. These systems consist of a plasmid with the transgenes to be integrated flanked by transposon sequences that play essential structural roles in the excision and integration processes along with a source of transposase. Transposase can be provided indirectly by simultaneously introducing either a functional transposase transcription unit or functional transposase mRNA with the vector. Transposase can, in some cases, be directly provided with the vector.

transposons is simple and because transposition requires little more than active transposase and a DNA target, transposon-based gene vectors have been conveniently assembled into binary vector systems with broad host ranges. Binary vector systems consist of a vector, comprising the inverted terminal repeats of the transposon and the transgenes to be introduced into the target genome and a plasmid containing the transposon-specific transposase open reading frame under regulatory control of a strong promoter that is active in cells of the germline (Fig. 1.3). Mixtures of these two plasmids are introduced into the appropriate germline stem cells where the transposase gene is transiently expressed and the resulting transposase protein mobilizes the accompanying vector (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Variations of this binary vector/helper plasmid strategy are possible, including, for example, co-injection of transposon-specific transposase mRNA with the vector-containing plasmids in lieu of 'helper' plasmids, a modification that reportedly increases transformation efficiencies (Kapetanaki *et al*., 2002) (Fig. 1.3). Also, purified transposase protein has been co-injected with vector DNA, precluding the need for any transposase gene expression in developing embryos (Coates *et al*., 2000) (Fig. 1.3). Delivery of vector systems to insect germline stem cells or developing gametes is accomplished almost exclusively by microinjecting young embryos with a mixture of the system's two components and consequently this can limit the applicability of the technology, since microinjecting embryos may not be feasible in some species of insect. Alternative delivery methods have been reported (Baldarelli and Lengyel, 1990; Mialhe and Miller, 1994; Yuen *et al*., 2008; Chaverra-Rodriguez *et al*., 2018; Lule-Chavez *et al*., 2021).

The development of transposon-based transgenic technology for *D. melanogaster* enabled this species to become a powerful model system for discovering and understanding genes and gene functions. Matthews and Vosshall (2020) recently recognized the continued importance of transgenic technologies in their consideration of how an organism can become a 'model' system and the need to move beyond established model systems. Transposon-based transgenic technologies can be foundational in efforts to develop new model systems, because their application does not depend on having a well-annotated genome.

1.5 Transposons as Technology Platforms

While delivering transgenes into genomes has been used extensively for structure/function studies of individual genes, transposons have also served as platforms for multiple functional genomic technologies that greatly extend their utility and importance. Some of those technologies include modular systems for gene expression, cell ablation, gene silencing, site-specific recombination and gene sensors.

1.5.1 Gene expression

Modular gene expression systems consist of an enhancer protein expressing a transgene and a transgene whose expression is dependent upon the presence of the enhancer protein. Only when both the enhancer-regulated transgene and the enhancer protein are in the same cell will the transgene be expressed. There are a number of such systems (see Schetelig *et al*., Chapter 2, this volume) (GAL4/UAS, *tTa/TRE*, LexA/LexAop and QF/QUAS) that depend largely on transposon-based technologies for their implementation and they are being increasingly used in non-model insects. By creating and maintaining collections of transgenic insects with different patterns of enhancer protein expression, a single transgene regulated by a single enhancer can be expressed in different spatial and temporal patterns by simply performing genetic crosses to place the transgene in the appropriate genetic background expressing the enhancer-binding protein in the desired pattern (Gatz and Quail, 1988; Bello *et al*., 1998; Baron and Bujard, 2000; Stebbins *et al*., 2001; Duffy,

2002; Imamura *et al.*, 2003; Lycett *et al.*, 2004; Karasaki *et al.*, 2009; Schetelig *et al.*, 2009; Schinko *et al.*, 2010; Kokoza and Raikhel, 2011; Lynd and Lycett, 2012; O'Brochta *et al.*, 2012; Schetelig and Handler, 2012; Riabinina *et al.*, 2015; Riabinina and Potter, 2016; Adolfi *et al.*, 2018; Driesschaert *et al.*, 2021; Poulton *et al.*, 2021).

1.5.2 Cell ablation

Transposon-based gene vectors are also used to introduce transgenes which, when expressed, will cause cell death – a technique called genetic cell ablation. Regulating cell death genes or genes encoding toxins using versatile modular expression systems allows great precision in ablating cells. Cell ablation by transgene expression is now just one of many options available for ablating cells (Kunes and Steller, 1991; Sentry *et al.*, 1993; Liu *et al.*, 2019).

Intentionally misexpressing genes with respect to their normal temporal and spatial patterns of expression can be a useful genetic approach for determining gene function (Feiler *et al.*, 1988; Rorth, 1996; Huang and Rubin, 2000; Foulger *et al.*, 2001; Huffman *et al.*, 2001; Zhang *et al.*, 2006; Stofanko *et al.*, 2008; Paik *et al.*, 2012; Prelich, 2012; Schinko *et al.*, 2012; Jezzini *et al.*, 2018; Meyer-Nava *et al.*, 2020). Inferring gene function from phenotypes arising from transgene misexpression can be difficult but it does represent a viable approach to the study of gene networks and the relationship between specific genes and particular phenotypes. The availability of transposon gene vectors and robust modular gene transgene expression systems is making this approach available in a wide range of insect systems (Kiya *et al.*, 2014; Kitzmann *et al.*, 2017).

1.5.3 Gene silencing

Transgene-based gene silencing of specific genes at particular times and in particular cells during development is a powerful genetic technology for studying the roles of genes that function at different stages in an organism's life cycle. Pleiotropy can complicate genetic analyses, but gene silencing technologies based on the expression of transgenes triggering RNA interference (RNAi) or components of RNA-guided DNA endonucleases (CRISPRi) are two technologies that can reveal and help understand pleiotropy. Transposon technologies have been key to the implementation of these technologies (Dietzl *et al.*, 2007; Perrimon *et al.*, 2010; Ghosh *et al.*, 2016; Heigwer *et al.*, 2018; Qiao *et al.*, 2018; Wang *et al.*, 2019; Xu *et al.*, 2019). Transgenic RNAi and CRISPRi silencing, while used mostly in *D. melanogaster*, have been applied in other insects (Franz *et al.*, 2006; Lee *et al.*, 2012; Wang *et al.*, 2019; Williams *et al.*, 2020).

Transposon-based vectors are also used to introduce the components of site-specific recombination systems such as the Flp/FRT, Cre/Lox and ΦC31 systems (see Ahmed and Wimmer, Chapter 5, this volume), which are used for post-integration genome modifications and for site-specific transgene integration (Venken and Bellen, 2005, 2007; Venken *et al.*, 2016). These systems have been used in a variety of ways in insects: to selectively remove components of a transgene cassette initially introduced into the genome of *D. melanogaster*, *B. mori*, *Ae. aegypti* or *C. capitata* (Jasinskiene *et al.*, 2003; Venken and Bellen, 2007; Schetelig *et al.*, 2009, 2011; Long *et al.*, 2012), to create chromosome deletions in *Drosophila* (Cook *et al.*, 2012) and to insert exogenous DNA sequences into specific locations within *D. melanogaster* and other species (Horn and Handler, 2005; Bateman *et al.*, 2006; Venken *et al.*, 2011; Yonemura *et al.*, 2012; Long *et al.*, 2015; Ahmed *et al.*, 2020). These site-specific recombination systems and their applications are discussed in more detail elsewhere in this book (see Ahmed and Wimmer, Chapter 5, this volume)

1.5.4 Genetic sensors

Transposon gene vectors, upon integration, can in some cases be induced to transpose further if the appropriate functional transposase

is provided. The potential of transposon gene vectors to remobilize and their rates of remobilization are element and host-species specific but when gene-vector remobilization within genomes is efficient, transposons can be created to carry genetic sensors that can be used to identify various features of genomes at or near the site of integration.

One of the first sensor technologies constructed with eukaryotic transposons was enhancer detectors. These sensors consist of reporter genes (e.g. *β galactosidase* (*lacZ*), *Gal4 transcription factor* (*Gal4*), fluorescent proteins) that are under the regulatory control of a minimal promoter, resulting in only basal levels of expression, and whose activity is insufficient to result in detectable levels of gene product and a visible phenotype (Brand and Perrimon, 1993; Singh, 1995; Kvon, 2015). Only when the sensor-containing transposon integrates into a region of the genome under the influence of an active enhancer are detectable levels of reporter gene expression attained. The temporal and spatial patterns of reporter gene expression are identical to those of the genes whose expression is normally regulated by the enhancer. Therefore, inducing remobilization of the transposon by providing transposase allows the genome to be scanned for the presence of regulatory elements (enhancers) (Kvon, 2015). If the enhancer-sensor is the gene for the transcription factor Gal4, then the regulatory sequences detected by the element can be used subsequently to drive the expression of any other transgene present within the same genome under the regulatory control of the Gal4-responsive *UAS*-containing promoter. Enhancer-traps with *Gal4* have been conducted extensively in *D. melanogaster*, providing researchers with a rich resource for investigating development and manipulating gene expression (Manseau *et al.*, 1997; Phelps and Brand, 1998). Because identifying, physically isolating and characterizing gene regulatory elements (promoters and enhancers) is challenging, enhancer-trapping technology permits these elements to be detected and then used to regulate the expression of any transgene without the need to clone or physically

characterize such elements (Kvon, 2015). Enhancer-trapping has been used in *T. castaneum, B. mori* and *An. stephensi* in addition to *D. melanogaster* (Uchino *et al.*, 2008; Schinko *et al.*, 2010; O'Brochta *et al.*, 2011; O'Brochta *et al.*, 2012; Lynd *et al.*, 2013).

Transposons can also be created that carry sensors capable of reporting on the presence of actively transcribed genes. These so-called gene-trap elements consist of a transposon with the open reading frame of a reporter gene (e.g. *EGFP*) or a transcription factor such as *Gal4* lacking any 5′ regulatory elements. Instead, a 3′ splice-acceptor site is attached to the 5′ end of the open reading frame. When the gene-trap element integrates into the intron of a gene, an alternate splicing event can occur leading to the expression of a fusion protein consisting partly of the target gene product and the fluorescent reporter protein (Lukacsovich *et al.*, 2001; Stanford *et al.*, 2001) or Gal4 (Gnerer *et al.*, 2015; Nagarkar-Jaiswal *et al.*, 2015b; Li-Kroeger *et al.*, 2018). The pattern of fusion protein expression will parallel that of the target gene, making a powerful means by which genes can be identified based on temporal and spatial patterns of gene expression. The transposon containing this gene sensor will be located in an intron of the target gene, and isolation and identification of the integration site enables the target gene to be subsequently isolated and identified. With this strategy, genes can be identified based on observable patterns of gene expression, which can provide invaluable information regarding the function of the gene. It should be noted that if these alternative splice events involving the gene-trap element are preferred, then little or no normal gene product will be produced, resulting in a mutant phenotype. So, gene- trap events can also be mutagenic and this is an added benefit of this technology. To date, gene-trap technology has been deployed mainly in *D. melanogaster* although it has been developed in *An. stephensi* (Lukacsovich *et al.*, 2001; Bonin and Mann, 2004; O'Brochta *et al.*, 2012).

Transposons can also be used to sense the presence of active promoters. Promoter-trap elements contain a reporter gene lacking any 5′ regulatory sequences, making it incapable

of being expressed except under special circumstances. The reporter gene will only be expressed when the element inserts 3′ of an active promoter and within the 5′ untranslated region or the first exon (Larsen *et al*., 2006). The use of this technology in insects has only been reported in *D. melanogaster* but is feasible in insects where enhancer- and gene-trap technology is available, including *T. castaneum*, *B. mori* and *An. stephensi*.

While gene-trap strategies based on alternate splicing and the expression of fusion proteins provide valuable information for understanding gene function, such as temporal and spatial patterns of expression within the organism, little information is actually obtained concerning the function of the target protein. Transposon-based protein-traps have been devised that permit the subcellular localization of the target gene product (Fedorova and Dorogova, 2020). Protein-traps in *Drosophila* usually consist of transposons carrying a promoterless fluorescent protein-coding region flanked by a functional 3′ splice acceptor and a 5′ splice donor (Larsen *et al*., 2006; Buszczak *et al*., 2007; Quinones-Coello *et al*., 2007) . When the protein-trap element integrates into the intron of an expressed gene, the exon containing the fluorescent protein-coding region is spliced into the target protein. Because the target protein will contain all signal sequences determining subcellular localization, an accurate representation of the target protein's distribution will result, providing key information about the function of the protein (Morin *et al*., 2001). The use of this technology in insects has only been reported in *D. melanogaster* but, given the successful development of gene- and enhancer-trapping technologies in *T. castaneum*, *B. mori* and *An. stephensi*, it should be highly feasible to implement protein-trap technology in these insects.

1.6 Hybrid Transposase Systems for Precision Integration

Altering the insertional profile of cut-and-paste DNA transposon vectors has been of interest as a means of mitigating undesirable mutagenic effects resulting from random integration patterns of most native cut-and-paste DNA transposons. Efforts to date have followed a strategy involving tethering of a secondary DNA-binding domain to the transposase protein with the intention of redirecting the transposon to specific target sites (Fig. 1.4). Maragathavally *et al*. (2006) fused the Gal4 DNA-binding protein to the *Tc1/mariner*-type element *Mos1* and *piggyBac* transposases. They reported a change in the targeting profile of these chimeric systems with insertions occurring proximal to Gal4 DNA recognition sequences in approximately 25% of the insertions (Maragathavally *et al*., 2006). Yant *et al*. (2007), working with *Sleeping Beauty* transposase tethered to the Gal4 DNA-binding domain and the zinc-finger protein EC2 in human cells, found biased targeting of *Sleeping Beauty* vectors. Owens *et al*. (2012, 2013) fused a transcription activator-like effector (TALE) protein to *piggyBac* transposase and reported biased insertion correlated with the TALE's target sequence. Insertions were found within 250 bp of the TALE's target sequence (Owens *et al*., 2012, 2013). Hew *et al*. (2019) and Goshayeshi *et al*. (2021) reported similar results. Luo *et al*. (2017) tethered zinc-finger proteins, TALEs, SpyCas9 and dSpyCas9 to *piggyBac* transposase and found that some zinc-finger proteins and TALEs increased transgene insertion specificity in human cells. More recently, the specific DNA-binding properties of Cas proteins have also been used in attempts to increase the precision of transposase-based integration of transgenes. Bhatt and Chalmers (2019) reported that dCas9–*Hsmar1* hybrid transposase resulted in 50% of *in vitro* transpositions into the dCas9-targeted site (dCas9 is a catalytically inactive – dead – Cas9). Chen and Wang (2019) created what they described as a Cas-Transposon (CasTn) in which they fused *Himar1* transposase to dCas9 that increased the insertion frequency into a single targeted TA dinucleotide more than 300 times compared with when the unmodified transposase was used (Chen and Wang, 2019). Kovac *et al*. (2020) tethered dCas9 to *Sleeping Beauty* transposase

4   **(A)**

(B)
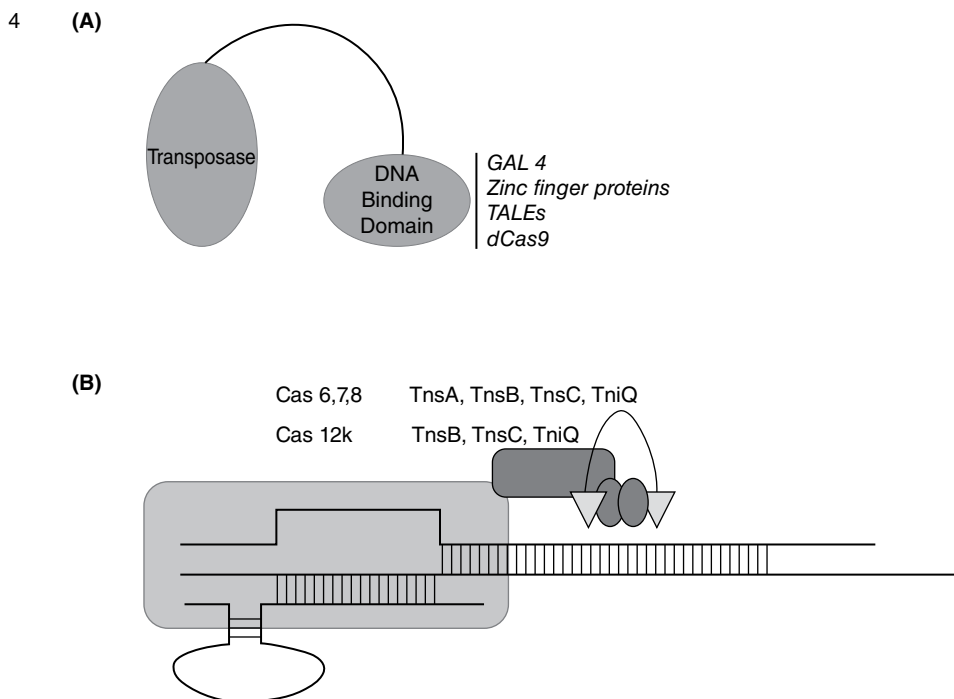
Fig. 1.4. **(A) A general strategy used to increase the precision of transposases.** Transposase is tethered to highly specific DNA-binding proteins including GAL4, specific zinc-finger proteins, transcription activator-like effectors (TALEs) and Cas9 protein lacking DNA endonuclease activity (dCas9). **(B) Two Cas-transposon systems in which the transposon integration site can be programmed using a guide RNA.**

and reported successfully directing *Sleeping Beauty* vector integration into regions of the human genome that were otherwise refractory to *Sleeping Beauty* insertions. While these systems have not been tested in insects or insect cells, with the exception of *Sleeping Beauty*, all of the transposon systems used are known to be or are very likely to be active in insects.

1.7   CRISPR-associated Transposases

Gene editing systems such as CRISPR/Cas (clustered regularly interspaced short-palindromic repeat/CRISPR-associated protein) can be used to place transgenes in genomes with high precision but at low efficiency, while transposon vectors can integrate

even large transgenes with relatively high efficiency but with low precision (see Concha and Papa, Chapter 7, this volume). Gene editing systems rely on DNA repair pathways such as homology-directed repair (HDR) following double-strand DNA cleavage, a process active in dividing cells. Transposon integration relies on a different mechanism that does not involve double-strand DNA cleavage and is not limited to dividing cells. As described in the previous section, there is interest in combining the best of transposon integration with gene editing precision. Recently a new class of transposon from bacteria was described that resembles *Tn*7, a transposon originally isolated from *Escherichia coli,* but which encoded CRISPR/Cas systems (Kapitonov *et al.*, 2016; Peters *et al.*, 2017). Target site integration of these transposons is determined by a guide RNA associated with the Cas protein that is encoded

within the transposon (Fig. 1.4). *Tn6677*, a *Tn7-Cas* transposon from *Vibrio cholerae,* and a *Tn7-Cas* transposon from *Scytonema homanni* have both been shown to be experimentally programmable transposon gene vectors that are functional in *E. coli* (Klompe *et al*., 2019; Strecker *et al*., 2019). In each case integration occurred at a fixed distance from the target sequence determined by the guide RNA (Fig. 1.4). Although this technology has only been tested and validated to date in *E. coli*, there is sufficient interest in these technologies across various sectors of biotechnology research and development to ensure that their full potential as useful eukaryote programmable integrase systems will be thoroughly explored. They could eventually become important tools for insect scientists.

1.8   Conclusion

Transposons are vital platforms upon which insect genetic technologies are being constructed. To date, transposons had been successfully demonstrated to have germline mobility in 53 different species of insect and the use of transposon-based technologies in insects will continue. The current collection of transposons with confirmed activity in heterologous insect germlines includes a diverse collection of elements. While certain elements have become extremely popular, no single element is likely to serve all of the needs of a given research community, as has been aptly demonstrated during the study of *Drosophila*. Having multiple elements with excellent functionality in a broad range of species is highly advantageous. The discovery and development of new programmable integrases promise to increase the utility of transposons as tools of genetics research and development. If new methods and strategies for delivering these systems to insect germ cells can be developed that do not rely on embryo microinjection, the many technologies described here will become more widely available and routinely applied in a larger number of species. Accelerated advances in insect functional genomics are expected to follow.

References

Adolfi, A., Pondeville, E., Lynd, A., Bourgouin, C. and Lycett, G.J. (2018) Multi-tissue GAL4-mediated gene expression in all *Anopheles gambiae* life stages using an endogenous polyubiquitin promoter. *Insect Biochemistry and Molecular Biology* 96, 1–9.

Ahmed, H.M.M., Heese, F. and Wimmer, E.A. (2020) Improvement on the genetic engineering of an invasive agricultural pest insect, the cherry vinegar fly, *Drosophila suzukii. BMC Genetics* 21, 13.

Allen, M.L., O'Brochta, D.A., Atkinson, P.W. and Levesque, C.S. (2001) Stable, germ-line transformation of *Culex quinquefasciatus* (Diptera: Culicidae). *Journal of Medical Entomology* 38, 701–710.

Allen, M.L., Handler, A.M., Berkebile, D.R. and Skoda, S.R. (2004) *piggyBac* transformation of the New World screwworm, *Cochliomyia hominivorax*, produces multiple distinct mutant strains. *Medical and Veterinary Entomology* 18, 1–9.

Amberger, M. and Ivics, Z. (2020) Latest advances for the *Sleeping Beauty* transposon system: 23 years of insomnia but prettier than ever. *Bioessays* 42, 14.

Arensburger, P., Hice, R.H., Zhou, L.Q., Smith, R.C., Tom, A.C. *et al.* (2011) Phylogenetic and functional characterization of the *hAT* transposon superfamily. *Genetics* 188, 45–83.

Arensburger, P., Orsetti, J., Kim, Y.-J., Aluvihare, C. and O'Brochta, D.A. (2005) A new active transposable element, *Herves*, from the African malaria mosquito *Anopheles gambiae*. *Genetics* 169, 697–708.

Asadi, R., Elaini, R., Lacroix, R., Ant, T., Collado, A. *et al.* (2020) Preventative releases of self-limiting *Ceratitis capitata* provide pest suppression and protect fruit quality in outdoor netted cages. *International Journal of Pest Management* 66, 182–193.

Atkinson, P.W., Warren, W.D. and O'Brochta, D.A. (1993) The *hobo* transposable element of *Drosophila* can be cross-mobilized in houseflies and excises like the *Ac* element of maize. *Proceedings of the National Academy of Sciences USA* 90, 9693–9697.

Baldarelli, R.M. and Lengyel, J.A. (1990) Transient expression of DNA after ballistic introduction into *Drosophila* embryos. *Nucleic Acids Research* 18, 5903–5904.

Baron, U. and Bujard, H. (2000) Tet repressor-based system for regulated gene expression in eukaryotic cells: Principles and advances. In: Thorner, J., Emr, S.D. and Abelson, J.N. (eds) *Complications of Chimeric Genes and Hybrid Proteins, Parr B: Cell Biology and Physiology.* Vol. 327 in book series *Methods in Enzymology*. Academic Press, New York, pp. 401–421.

Bateman, J.R., Lee, A.M. and Wu, C.T. (2006) Site-specific transformation of *Drosophila* via phiC31 integrase-mediated cassette exchange. *Genetics* 173, 769–777.

Bellen, H.J., Levis, R.W., Liao, G., He, Y., Carlson, J.W. *et al.* (2004) The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila* genes. *Genetics* 167, 761–781.

Bellen, H.J., Levis, R.W., He, Y.C., Carlson, J.W., Evans-Holm, M. *et al.* (2011) The *Drosophila* gene disruption project: Progress using transposons with distinctive site specificities. *Genetics* 188, 731–U341.

Bello, B., Resendez-Perez, D. and Gehring, W.J. (1998) Spatial and temporal targeting of gene expression in *Drosophila* by means of a tetracycline-dependent transactivator system. *Development* 125, 2193–2202.

Berghammer, A.J., Klingler, M. and Wimmer, E.A. (1999) Genetic techniques: a universal marker for transgenic insects. *Nature* 402, 370.

Bhatt, S. and Chalmers, R. (2019) Targeted DNA transposition in vitro using a dCas9-transposase fusion protein. *Nucleic Acids Research* 47, 8126–8135.

Blackman, R.K. and Gelbart, W.M. (1989) The transposable element *hobo* of *Drosophila melanogaster*. In: Berg, D.E. and How, M.M. (eds) *Mobile DNA*. American Society for Microbiology Publications, Washington, DC, pp. 523–529.

Bonin, C.P. and Mann, R.S. (2004) A *piggyBac* transposon gene trap for the analysis of gene expression and function in *Drosophila*. *Genetics* 167, 1801–1811.

Bossin, H., Furlong, R.B., Gillett, J.L., Bergoin, M. and Shirk, P.D. (2007) Somatic transformation efficiencies and expression patterns using the *JcDNV* and *piggyBac* transposon gene vectors in insects. *Insect Molecular Biology* 16, 37–47.

Bossin, H.C., Thailayil, J., Catteruccia, F., Benton, J.P., Crisanti, A. *et al.* (2006) First *Anopheles arabiensis* germline transformation: toward the development of a transgenic genetic sexing strain. *American Journal of Tropical Medicine and Hygiene* 75, 66–66.

Bouallègue, M., Rouault, J.-D., Hua-Van, A., Makni, M. and Capy, P. (2017) Molecular evolution of *piggyBac* superfamily: from selfishness to domestication. *Genome Biology and Evolution* 9, 323–339.

Bouuaert, C.C. and Chalmers, R.M. (2010) Gene therapy vectors: the prospects and potentials of the cut-and-paste transposons. *Genetica* 138, 473–484.

Brand, A.H. and Perrimon, N. (1993) Targeted gene-expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118, 401–415.

Brennan, M.D., Rowan, R.G. and Dickinson, W.J. (1984) Introduction of a functional *P* element into the germ-line of *Drosophila hawaiiensis*. *Cell* 38, 147–151.

Bryan, G.J., Jacobson, J.W. and Hartl, D.L. (1987) Heritable somatic excision of a *Drosophila* transposon. *Science* 235, 1636–1638.

Buszczak, M., Paterno, S., Lighthouse, D., Bachman, J., Planck, J *et al.* (2007) The Carnegie protein trap library: a versatile tool for *Drosophila* developmental studies. *Genetics* 175, 1505–1531.

Cain, A.K., Barquist, L., Goodman, A.L., Paulsen, I.T., Parkhill, J. and van Opijnen, T. (2020) A decade of advances in transposon-insertion sequencing. *Nature Reviews Genetics* 21, 526–540.

Caroti, F., Urbansky, S., Wosch, M. and Lemke, S. (2015) Germ line transformation and in vivo labeling of nuclei in Diptera: report on *Megaselia abdita* (Phoridae) and *Chironomus riparius* (Chironomidae). *Development Genes and Evolution* 225, 179–186.

Catteruccia, F., Nolan, T., Blass, C., Muller, H.M., Crisanti, A. *et al.* (2000) Toward *Anopheles* transformation: *Minos* element activity in anopheline cells and embryos. *Proceedings of the National Academy of Sciences USA* 97, 2157–2162.

Chaverra-Rodriguez, D., Macias, V.M., Hughes, G.L., Pujhari, S., Suzuki, Y. *et al.* (2018) Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing. *Nature Communications* 9, 11.

Chen, S.P. and Wang, H.H. (2019) An engineered Cas-transposon system for programmable and site-directed DNA transpositions. *CRISPR Journal* 2, 376–394.

Chu, F., Klobasa, W., Wu, P., Pinzi, S., Grubbs, N. *et al.* (2017) Germline transformation of the western corn rootworm, *Diabrotica virgifera virgifera*. *Insect Molecular Biology* 26, 440–452.

Chu, F.C., Klobasa, W., Grubbs, N. and Lorenzen, M.D. (2018) Development and use of a *piggyBac*-based jumpstarter system in *Drosophila suzukii*. *Archives of Insect Biochemistry and Physiology* 97, e21439.

Coates, C.J., Jasinskiene, N., Miyashiro, L. and James, A.A. (1998) *Mariner* transposition and transform-ation of the yellow fever mosquito, *Aedes aegypti*. *Proceedings of the National Academy of Sciences USA* 95, 3748–3751.

Coates, C.J., Jasinskiene, N., Morgan, D., Tosi, L.R.O., Beverley, S.M. and James, A.A. (2000) Puri-fied mariner (*Mos1*) transposase catalyzes the integration of marked elements into the germ-line of the yellow fever mosquito, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* 30, 1003–1008.

Concha, C., Belikoff, E.J., Carey, B.L., Li, F., Schiemann, A.H. and Scott, M.J. (2011) Efficient germ-line transformation of the economically important pest species *Lucilia cuprina* and *Lucilia sericata* (Diptera, Calliphoridae). *Insect Biochemistry and Molecular Biology* 41, 70–75.

Condon, K.C., Condon, G.C., Dafa'alla, T.H., Forrester, O.T., Phillips, C.E. *et al.* (2007) Germ-line transformation of the Mexican fruit fly. *Insect Molecular Biology* 16, 573–580.

Cook, R.K., Christensen, S.J., Deal, J.A., Coburn, R.A., Deal, M.E. *et al.* (2012) The generation of chromosomal deletions to provide extensive coverage and subdivision of the *Drosophila melanogaster* genome. *Genome Biology* 13, Article 21.

DeNicola, G.M., Karreth, F.A., Adams, D.J. and Wong, C.C. (2015) The utility of transposon mutagenesis for cancer studies in the era of genome editing. *Genome Biology* 16, Article 229.

DeVault, J.D., Hughes, K.J., Leopold, R.A., Johnson, O.A. and Narang, S.K. (1996) Gene transfer into corn earworm (*Helicoverpa zea*) embryos. *Genome Research* 6, 571–579.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y. *et al.* (2007) A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature* 448, 151–151.

Drabek, D., Zagoraiou, L., deWit, T., Langeveld, A., Roumpaki, C. *et al.* (2003) Transposition of the *Drosophila hydei Minos* transposon in the mouse germ line. *Genomics* 81, 108–111.

Driesschaert, B., Mergan, L. and Temmerman, L. (2021) Conditional gene expression in invertebrate animal models. *Journal of Genetics and Genomics* 48, 14–31.

Duffy, J.B. (2002) GAL4 system in *Drosophila*: a fly geneticist's Swiss army knife. *Genesis* 34, 1–15.

Eckermann, K.N., Ahmed, H.M.M., KaramiNejadRanjbar, M., Dippel, S., Ogaugwu, C.E. *et al.* (2018) Hyperactive *piggyBac* transposase improves transformation efficiency in diverse insect species. *Insect Biochemistry and Molecular Biology* 98, 16–24.

Fedorova, S.A. and Dorogova, N.V. (2020) Protein trap: a new Swiss army knife for geneticists? *Molecular Biology Reports* 47, 1445–1458.

Feiler, R., Harris, W.A., Kirschfeld, K., Wehrhahn, C. and Zuker, C.S. (1988) Targeted misexpression of a Drosophila *opsin* gene leads to altered visual function. *Nature* 333, 737–741.

Ferguson, H.J., Neven, L.G., Thibault, S.T., Mohammed, A. and Fraser, M. (2011) Genetic transform-ation of the codling moth, *Cydia pomonella* L., with *piggyBac EGFP*. *Transgenic Research* 20, 201–214.

Finokiet, M., Goni, B. and Loreto, E.L.S. (2007) Genetic transformation of *Drosophila willistoni* using *piggyBac* transposon and GFP. *Brazilian Archives of Biology and Technology* 50, 8.

Foulger, R.E., Goberdhan, D.C.I. and Wilson, C. (2001) A misexpression screen to identify novel regulators of growth and proliferation in *Drosophila*. *Developmental Biology* 235, 252–253.

Franz, A.W.E., Sanchez-Vargas, I., Adelman, Z.N., Blair, C.D., Beaty, B.J. *et al.* (2006) Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. *Proceed-ings of the National Academy of Sciences USA* 103, 4198–4203.

Franz, G. and Savakis, C. (1991) *Minos*, a new transposable element from *Drosophila hydei*, is a member of the *Tc*1-like family of transposons. *Nucleic Acids Research* 19, 6646.

Franz, G., Loukeris, T.G., Dialektaki, G., Thompson, C.R.L. and Savakis, C. (1994) Mobile *Minos* elements from *Drosophila hydei* encode a two-exon transposase with similarity to the *paired* DNA-binding domain. *Proceedings of the National Academy of Sciences USA* 91, 4746–4750.

Fraser, M.J., Smith, G.E. and Summers, M.D. (1983) The acquisition of host cell DNA sequences by baculo-viruses: relation between host DNA insertions and FP mutants of *Autographa californica* and *Galleria mellonella* NPVs. *Journal of Virology* 47, 287–300.

Fraser, M.J., Brusca, J.S., Smith, G.E. and Summers, M.D. (1985) Transposon-mediated mutagenesis of baculoviruses. *Virology* 145, 356–361.

Garza, D., Medhora, M., Koga, A. and Hartl, D.L. (1991) Introduction of the transposable element *mariner* into the germline of *Drosophila melanogaster*. *Genetics* 128, 303–310.

Gatz, C. and Quail, P.H. (1988) *Tn10*-encoded tet repressor can regulate an operator-containing plant promoter. *Proceedings of the National Academy of Sciences USA* 85, 1394–1397.

Genc, H., Schetelig, M.F., Nirmala, X. and Handler, A.M. (2016) Germline transformation of the olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), with a *piggyBac* transposon vector. *Turkish Journal of Biology* 40, 845–855.

Ghosh, S., Tibbit, C. and Liu, J.L. (2016) Effective knockdown of *Drosophila* long non-coding RNAs by CRISPR interference. *Nucleic Acids Research* 44(9), e84

Gnerer, J.P., Venken, K.J.T. and Dierick, H.A. (2015) Gene-specific cell labeling using *MiMIC* transposons. *Nucleic Acids Research* 43, 13.

Gorman, K., Young, J., Pineda, L., Márquez, R., Sosa, N. *et al.* (2016) Short-term suppression of *Aedes aegypti* using genetic control does not facilitate *Aedes albopictus*. *Pest Management Science* 72, 618–628.

Goryshin, I.Y., Jendrisak, J., Hoffman, L.M., Meis, R. and Reznikoff, W.S. (2000) Insertional transposon mutagenesis by electroporation of released *Tn5* transposition complexes. *Nature Biotechnology* 18, 97–100.

Goshayeshi, L., Taemeh, S.Y., Dehdilani, N., Nasiri, M., Seno, M.M.G. and Dehghani, H. (2021) CRISPR/dCas9-mediated transposition with specificity and efficiency of site-directed genomic insertions. *FASEB Journal* 35(2), e21359.

Grossman, G.L., Rafferty, C.S., Clayton, J.R., Stevens, T.K., Mukabayire, O. and Benedict, M.Q. (2001) Germline transformation of the malaria vector, *Anopheles gambiae*, with the *piggyBac* transposable element. *Insect Molecular Biology* 10, 597–604.

Guimond, N., Bideshi, D.K., Pinkerton, A.C., Atkinson, P.W. and O'Brochta, D.A. (2003) Patterns of *Hermes* transposition in *Drosophila melanogaster*. *Molecular & General Genetics* 268, 779–790.

Handler, A.M. and Harrell, R.A. (1999) Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector. *Insect Molecular Biology* 8, 449–457.

Handler, A.M. and Harrell, R.A. (2001) Transformation of the Caribbean fruit fly, *Anastrepha suspensa*, with a *piggyBac* vector marked with polyubiquitin-regulated GFP. *Insect Biochemistry and Molecular Biology* 31, 199–205.

Handler, A.M. and McCombs, S.D. (2000) The *piggyBac* transposon mediates germ-line transformation in the Oriental fruit fly and closely related elements exist in its genome. *Insect Molecular Biology* 9, 605–612.

Handler, A.M. and O'Brochta, D.A. (2012) Transposable elements for insect transformation. In: Gilbert, L.I. (ed.) *Insect Molecular Biology and Biochemistry*. Academic Press, San Diego, California, pp. 90–133.

Handler, A.M. and Schetelig, M.F. (2020) The *hAT*-family transposable element, *hopper*, from *Bactrocera dorsalis* is a functional vector for insect germline transformation. *BMC Genetics* 21, 137.

Handler, A.M., McCombs, S.D., Fraser, M.J. and Saul, S.H. (1998) The lepidopteran transposon vector, *piggyBac*, mediates germ-line transformation in the Mediterranean fruit fly. *Proceedings of the National Academy of Sciences USA* 95, 7520–7525.

Hayes, F. (2003) Transposon-based strategies for microbial functional genomics and proteomics. *Annual Review of Genetics* 37, 3–29.

Hediger, M., Niessen, M., Wimmer, E.A., Dübendorfer, A.a.D. and Bopp, D. (2001) Genetic transformation of the housefly *Musca domestica* with the lepidopteran derived transposon *piggyBac*. *Insect Molecular Biology* 10, 113–119.

Heigwer, F., Port, F. and Boutros, M. (2018) RNA Interference (RNAi) Screening in *Drosophila*. *Genetics* 208, 853–874.

Heinrich, J.C., Li, X., Henry, R.A., Haack, N., Stringfellow, L. *et al.* (2002) Germ-line transformation of the Australian sheep blowfly *Lucilia cuprina*. *Insect Molecular Biology* 11, 1–10.

Hew, B.E., Sato, R., Mauro, D., Stoytchev, I. and Owens, J.B. (2019) RNA-guided *piggyBac* transposition in human cells. *Synthetic Biology* 4(1), ysz018.

Holtzman, S., Miller, D., Eisman, R., Kuwayama, H., Niimi, T. and Kaufman, T.C. (2010) Transgenic tools for members of the genus *Drosophila* with sequenced genomes. *Fly* 4, 1–14.

Horn, C. and Handler, A.M. (2005) Site-specific genomic targeting in *Drosophila*. *Proceedings of the National Academy of Sciences USA* 102, 12483–12488.

Huang, A.M. and Rubin, G.M. (2000) A misexpression screen identifies genes that can modulate RAS1 pathway signaling in *Drosophila melanogaster*. *Genetics* 156, 1219–1230.

Huffman, D., Reiter, L.T. and Bier, E. (2001) Misexpression of 50 uncharacterized Drosophila homologs to human disease genes reveals insight into gene function. *American Journal of Human Genetics* 69, 469–469.

Imamura, M., Junichi, N., Inoue, S., Quan, G.X., Kanda, T. and Tamura, T. (2003) Targeted gene expression using the GAL4/UAS system in the silkworm *Bombyx mori*. *Genetics* 165, 1329–1340.

Ivics, Z. and Izsvak, Z. (2010) The expanding universe of transposon technologies for gene and cell engineering. *Mobile DNA* 1, 25.

Jasinskiene, N., Coates, C.J., Benedict, M.Q., Cornel, A.J., Rafferty, C.S. *et al.* (1998) Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the *Hermes* element from the housefly. *Proceedings of the National Academy of Sciences USA* 95, 3743–3747.

Jasinskiene, N., Coates, C.J., Ashikyan, A. and James, A.A. (2003) High efficiency, site-specific excision of a marker gene by the phage P1 cre-loxP system in the yellow fever mosquito, *Aedes aegypti*. *Nucleic Acids Research* 31, e147.

Jezzini, S.H., Merced, A. and Blagburn, J.M. (2018) Shaking-B misexpression increases the formation of gap junctions but not chemical synapses between auditory sensory neurons and the giant fiber of *Drosophila melanogaster*. *PLoS ONE* 13, 17.

Kapetanaki, M.G., Loukeris, T.G., Livadaras, I. and Savakis, C. (2002) High frequencies of *Minos* transposon mobilization are obtained in insects by using in vitro synthesized mRNA as a source of transposase. *Nucleic Acids Research* 30, 3333–3340.

Kapitonov, V.V. and Jurka, J. (2008) A universal classification of eukaryotic transposable elements implemented in Repbase. *Nature Reviews Genetics* 9, 411–412.

Kapitonov V.V., Makarova, K.S., Koonin, E.V. and Zhulin, I.B. (2016) ISC, a novel group of bacterial and archaeal DNA transposons that encode Cas9 homologs. *Journal of Bacteriology* 198, 797–807.

Karasaki, N., Mon, H., Takahashi, M., Lee, J.M., Koga, K. *et al.* (2009) Establishment of tetracycline-inducible gene expression systems in the silkworm, *Bombyx mori*. *Biotechnology Letters* 31, 495–500.

Kawakami, K., Largaespada, D.A. and Ivics, Z. (2017) Transposons as tools for functional genomics in vertebrate models. *Trends in Genetics* 33, 784–801.

Kebriaei, P., Izsvk, Z., Narayanavari, S.A., Singh, H. and Ivics, Z. (2017) Gene therapy with the *Sleeping Beauty* transposon system. *Trends in Genetics* 33, 852–870.

Kempken, F. and Windhofer, F. (2001) The *hAT* family: a versatile transposon group common to plants, fungi, animals, and man. *Chromosoma* 110, 1–9.

Kidwell, M.G., Kidwell, J.F. and Sved, J.A. (1977) Hybrid dysgenesis in *Drosophila melanogaster*: a syndrome of aberrant traits including mutation, sterility and male recombination. *Genetics* 86(4), 813–833.

Kim, S.J., Sung, S.H., Seung, J.Y., Nam, H.Y., Kim, K.C. and Suh, D.S. (2003) Production of transgenic silkworm (*Bombyx mori* L.) by *P* element-mediated integration of recombinant luciferase with silkworm fibroin promoter. *Korean Journal of Genetics* 25, 57–62.

Kirby, J.R. (2007) In vivo mutagenesis using *EZ-Tn5*(TM). In: Hughes, K.T. and Maloy, S.R. (eds) *Advanced Bacterial Genetics: Use of Transposons and Phage for Genomic Engineering*, Vol. 421. Elsevier Academic Press, San Diego, California, pp. 17–21.

Kitzmann, P., Weisskopf, M., Schacht, M.I. and Bucher, G. (2017) A key role for *foxQ2* in anterior head and central brain patterning in insects. *Development* 144, 2969–2981.

Kiya, T., Morishita, K., Uchino, K., Iwami, M. and Sezutsu, H. (2014) Establishment of tools for neurogenetic analysis of sexual behavior in the silkmoth, *Bombyx mori*. *PLoS ONE* 9, 7.

Klompe, S.E., Vo, P.L.H., Halpin-Healy, T.S. and Sternberg, S.H. (2019) Transposon-encoded CRISPR–Cas systems direct RNA-guided DNA integration. *Nature* 571, 219–225.

Kokoza, V.A. and Raikhel, A.S. (2011) Targeted gene expression in the transgenic *Aedes aegypti* using the binary Gal4-UAS system. *Insect Biochemistry and Molecular Biology* 41, 637–644.

Kokoza, V., Ahmed, A., Wimmer, E.A. and Raikhel, A.S. (2001) Efficient transformation of the yellow fever mosquito *Aedes aegypti* using the *piggyBac* transposable element vector *pBac[3xP3-EGFP afm]*. *Insect Biochemistry and Molecular Biology* 31, 1137–1143.

Koukidou, M., Klinakis, A., Reboulakis, C., Zagoraiou, L., Tavernarakis, N. *et al.* (2006) Germ line transformation of the olive fly *Bactrocera oleae* using a versatile transgenesis marker. *Insect Molecular Biology* 15, 95–103.

Kovac, A., Miskey, C., Menzel, M., Grueso, E., Gogol-Doring, A. and Ivics, Z. (2020) RNA-guided retargeting of *Sleeping Beauty* transposition in human cells. *Elife* 9, 19.

Kumar, D., Anand, T., Talluri, T.R. and Kues, W.A. (2020) Potential of transposon-mediated cellular reprogramming towards cell-based therapies. *World Journal of Stem Cells* 12, 527–544.

Kunes, S. and Steller, H. (1991) Ablation of *Drosophila* photoreceptor cells by conditionsl expression of a toxin gene. *Genes and Development* 5, 970–983.

Kuwayama, H., Yaginuma, T., Yamashita, O. and Niimi, T. (2006) Germ-line transformation and RNAi of the ladybird beetle, *Harmonia axyridis*. *Insect Molecular Biology* 15, 507–512.

Kvon, E.Z. (2015) Using transgenic reporter assays to functionally characterize enhancers in animals. *Genomics* 106, 185–192.

Labbé, G.M.C., Nimmo, D.D. and Alphey, L. (2010) *piggyBac*- and *phiC31*-mediated genetic transformation of the Asian tiger mosquito, *Aedes albopictus* (Skuse) *PLoS Neglected Tropical Diseases* 4(8), e788.

Laptev, I., Raevskaya, N., Filimonova, N. and Sineoky, S. (2017) The *piggyBac* Transposon as a tool in genetic engineering. *Applied Biochemistry and Microbiology* 53, 874–881.

Larsen, C., Franch-Marro, X., Hartenstein, V., Alexandre, C. and Vincent, J.P. (2006) An efficient promoter trap for detection of patterned gene expression and subsequent functional analysis in *Drosophila*. *Proceedings of the National Academy of Sciences USA* 103, 17813–17817.

Lee, J.M., Kojin, Y., Tatsuke, T., Mon, H., Miyagawa, Y. and Kusakabe, T. (2012) RNA interference induction by long hairpin dsRNAs expressed from chromosomal DNA of *Bombyx mori* cells. *Journal of the Faculty of Agriculture Kyushu University* 57, 441–445.

Lee, P.T., Zirin, J., Kanca, O., Lin, W.W., Schulze, K.L. *et al.* (2018) A gene-specific T2A-GAL4 library for Drosophila. *Elife* 7, e35574.

Li, M.A., Turner, D.J., Ning, Z., Yusa, K., Liang, Q. *et al.* (2011) Mobilization of giant *piggyBac* transposons in the mouse genome. *Nucleic Acids Research* 39, e148–e148.

Li, N.N., Jin, K.R., Bai, Y.M., Fu, H.F., Liu, L. and Liu, B. (2020) *Tn5* transposase applied in genomics research. *International Journal of Molecular Sciences* 21(21), 8329.

Li-Kroeger, D., Kanca, O., Lee, P.T., Cowan, S., Lee, M.T. *et al.* (2018) An expanded toolkit for gene tagging based on *MiMIC* and scarless CRISPR tagging in Drosophila. *Elife* 7, 27.

Liu, D., Yan, S.C., Liu, D., Huang, Y.P., Tan, A.J. *et al.* (2012) Genetic transformation mediated by *piggyBac* in the Asian Corn Borer, *Ostrinia furnacalis* (Lepidoptera: Crambidae) *Archives of Insect Biochemistry and Physiology* 80, 140–150.

Liu, D.X. and Chalmers, R. (2014) Hyperactive *mariner* transposons are created by mutations that disrupt allosterism and increase the rate of transposon end synapsis. *Nucleic Acids Research* 42, 2637–2645.

Liu, F.M., Dai, S., Feng, D.C., Peng, X., Qin, Z.N. *et al.* (2019) Versatile cell ablation tools and their applications to study loss of cell functions. *Cellular and Molecular Life Sciences* 76, 4725–4743.

Liu, J.G., Qiao, L., Zhang, J.J., Chen, B. and He, Z.B. (2021) *piggyBac*-mediated germline transformation of the malaria mosquito *Anopheles sinensis* (Diptera: Culicidae). *Insect Science* 28, 1202–1206.

Lohe, A.R. and Hartl, D.L. (1996) Germline transformation of *Drosophila virilis* with the transposable element *mariner*. *Genetics* 143, 365–374.

Long, D.P., Zhao, A.C., Chen, X.J., Zhang, Y., Lu, W.J. *et al.* (2012) FLP recombinase-mediated site-specific recombination in silkworm, *Bombyx mori*. *PLoS ONE* 7, e40150.

Long, D.P., Lu, W.J., Zhang, Y.L., Bi, L.H., Xiang, Z.H. and Zhao, A.C. (2015) An efficient strategy for producing a stable, replaceable, highly efficient transgene expression system in silkworm, *Bombyx mori*. *Scientific Reports* 5, 14.

Loukeris, T.G., Arca, B., Livadaras, I., Dlalektaki, G. and Savakis, C. (1995a) Introduction of the transposable element *Minos* into the germ line of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences USA* 92, 9485–9489.

Loukeris, T.G., Livadaras, I., Arca, B., Zabalou, S. and Savakis, C. (1995b) Gene transfer into the Medfly, *Ceratitis capitata*, using a *Drosophila hydei* transposable element. *Science* 270, 2002–2005.

Lozovskaya, E.R., Nurminsky, D.I., Hartl, D.L. and Sullivan, D.T. (1996) Germline transformation of *Drosophila virilis* mediated by the transposable element *hobo*. *Genetics* 142, 173–177.

Lukacsovich, T., Asztalos, Z., Awano, W., Baba, K., Kondo, S. *et al.* (2001) Dual-tagging gene trap of novel genes in *Drosophila melanogaster*. *Genetics* 157, 727–742.

Lule-Chavez, A.N., Carballar-Lejarazu, R., Cabrera-Ponce, J.L., Lanz-Mendoza, H. and Ibarra, J.E. (2021) Genetic transformation of mosquitoes by microparticle bombardment. *Insect Molecular Biology* 30, 30–41.

Luo, W.T., Galvan, D.L., Woodard, L.E., Dorset, D., Levy, S. and Wilson, M.H. (2017) Comparative analysis of chimeric ZFP-, TALE- and Cas9-*piggyBac* transposases for integration into a single locus in human cells. *Nucleic Acids Research* 45, 8411–8422.

Lycett, G.J., Kafatos, F.C. and Loukeris, T.G. (2004) Conditional expression in the malaria mosquito *Anopheles stephensi* with Tet-On and Tet-Off systems. *Genetics* 167, 1781–1790.

Lynd, A. and Lycett, G.J. (2012) Development of the bi-partite Gal4-UAS system in the African malaria mosquito, *Anopheles gambiae*. *PLoS ONE* 7, e31552.

Lynd, A., Amenya, D. and Lycett, G. (2013) Utilization of the Gal4/UAS system for enhancer trapping in transgenic *Anopheles gambiae*. *Pathogens and Global Health* 107, 444–445.

Majumdar, S. and Rio, D.C. (2015) *P* transposable elements in *Drosophila* and other eukaryotic organisms. In: *Mobile DNA III*. ASM Press, Washington, DC, pp. 727–752.

Manseau, L., Baradaran, A., Brower, D., Budhu, A., Elefant, F. *et al.* (1997) GAL4 enhancer traps expressed in the embryo, larval brain, imaginal discs, and ovary of *Drosophila*. *Developmental Dynamics* 209, 310–322.

Maragathavally, K.J., Kaminski, J.M. and Coates, C.J. (2006) Chimeric *Mos1* and *piggyBac* transposases result in site-directed integration. *FASEB Journal* 20, 1880–1882.

Marcus, J.M., Ramos, D.M. and Monteiro, A. (2004) Germline transformation of the butterfly *Bicyclus anynana*. *Proceedings of the Royal Society of London Series B: Biological Sciences* 271, S263–S265.

Martins, S., Naish, N., Walker, A.S., Morrison, N.I., Scaife, S. *et al.* (2012) Germline transformation of the diamondback moth, *Plutella xylostella* L., using the *piggyBac* transposable element. *Insect Molecular Biology* 21, 414–421.

Matthews, B.J. and Vosshall, L.B. (2020) How to turn an organism into a model organism in 10 'easy' steps. *Journal of Experimental Biology* 223, 10.

McGinnis, W., Shermoen, A.W. and Beckendorf, S.K. (1983) A transposable element inserted just 5' to a *Drosophila* glue protein gene alters gene expression and chromatin structure. *Cell* 34, 75–84.

Medhora, M.M., MacPeek, A.H. and Hartl, D.L. (1988) Excision of the *Drosophila* transposable element *mariner*: identification and characterization of the *Mos* factor. *EMBO Journal* 7, 2185–2189.

Metaxakis, A., Oehler, S., Klinakis, A. and Savakis, C. (2005) *Minos* as a genetic and genomic tool in *Drosophila melanogaster*. *Genetics* 171, 571–581.

Meyer-Nava, S., Torres, A., Zurita, M. and Valadez-Graham, V. (2020) Molecular effects of dADD1 misexpression in chromatin organization and transcription. *BMC Molecular and Cell Biology* 21, 17.

Mialhe, E. and Miller, L.H. (1994) Biolistic techniques for transfection of mosquito embryos (*Anopheles gambiae*). *Biotechniques* 16, 924–931.

Michel, K., Stamenova, A., Pinkerton, A.C., Franz, G., Robinson, A.S. *et al.* (2001) *Hermes*-mediated germ-line transformation of the mediterranean fruit fly *Ceratitis capitata*. *Insect Molecular Biology* 10, 155–162.

Morin, X., Daneman, R., Zavortink, M. and Chia, W. (2001) A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in *Drosophila*. *Proceedings of the National Academy of Sciences USA* 98, 15050–15055.

Muñoz-López, M. and García-Pérez, J.L. (2010) DNA transposons: nature and applications in genomics. *Current Genomics* 11, 115–128.

Nagarkar-Jaiswal, S., DeLuca, S.Z., Lee, P.-T., Lin, W.-W., Pan, H. *et al.* (2015a) A genetic toolkit for tagging intronic *MiMIC* containing genes. *Elife* 4, e08469.

Nagarkar-Jaiswal, S., Lee, P.-T., Campbell, M.E., Chen, K., Anguiano-Zarate, S. *et al.* (2015b) A library of *MiMICs* allows tagging of genes and reversible, spatial and temporal knockdown of proteins in Drosophila. *Elife* 4, e05338.

Nakamura, T., Yoshizaki, M., Ogawa, S., Okamoto, H., Shinmyo, Y. *et al.* (2010) Imaging of transgenic cricket embryos reveals cell movements consistent with a syncytial patterning mechanism. *Current Biology* 20, 1641–1647.

Nolan, T., Bower, T.M., Brown, A.E., Crisanti, A. and Catteruccia, F. (2002) *piggyBac*-mediated germline transformation of the malaria mosquito *Anopheles stephensi* using the red fluorescent protein dsRED as a selectable marker. *Journal of Biological Chemistry* 277, 8759–8762.

O'Brochta, D.A. and Handler, A.M. (1988) Mobility of *P* elements in drosophilids and nondrosophilids. *Proceedings of the National Academy of Sciences USA* 85(16), 6052–6056.

O'Brochta, D.A., Warren, W.D., Saville, K.J. and Atkinson, P.W. (1996) *Hermes*, a functional non-drosophilid insect gene vector from *Musca domestica*. *Genetics* 142, 907–914.

O'Brochta, D.A., Atkinson, P.W. and Lehane, M.J. (2000) Transformation of *Stomoxys calcitrans* with a *Hermes* gene vector. *Insect Molecular Biology* 9, 531–538.

O'Brochta, D.A., Alford, R.T., Pilitt, K.L., Aluvihare, C.U. and Harrell, R.A. (2011) *piggyBac* transposon remobilization and enhancer detection in *Anopheles* mosquitoes. *Proceedings of the National Academy of Sciences USA* 108, 16339–16344.

O'Brochta, D.A., Pilitt, K.L., Harrell II, R.A., Aluvihare, C. and Alford, R.T. (2012) *Gal4*-based enhancer-trapping in the malaria mosquito *Anopheles stephensi*. *G3: Genes, Genomes, Genetics* 2, 1305–1315.

Owens, J.B., Mauro, D., Stoytchev, I., Bhakta, M.S., Kim, M.S. *et al.* (2013) Transcription activator like effector (TALE)-directed *piggyBac* transposition in human cells. *Nucleic Acids Research* 41, 9197–9207.

Owens, J.B., Urschitz, J., Stoytchev, I., Dang, N.C., Stoytcheva, Z. *et al.* (2012) Chimeric *piggyBac* transposases for genomic targeting in human cells. *Nucleic Acids Research* 40, 6978–6991.

Paik, D., Jang, Y.G., Lee, Y.E., Lee, Y.N., Yamamoto, R. *et al.* (2012) Misexpression screen delineates novel genes controlling Drosophila lifespan. *Mechanisms of Ageing and Development* 133, 234–245.

Pavlopoulos, A. and Averof, M. (2005) Establishing genetic transformation for comparative developmental studies in the crustacean *Parhyale hawaiensis*. *Proceedings of the National Academy of Sciences USA* 102, 7888–7893.

Pavlopoulos, A., Berghammer, A.J., Averof, M. and Klingler, M. (2004) Efficient transformation of the beetle *Tribolium castaneum* using the *Minos* transposable element: Quantitative and qualitative analysis of genomic integration events. *Genetics* 167, 737–746.

Peloquin, J.J., Thibault, S.T., Staten, R. and Miller, T.A. (2000) Germ-line transformation of pink bollworm (Lepidoptera: Gelechiidae) mediated by the *piggyBac* transposable element. *Insect Molecular Biology* 9, 323–333.

Perera, O.P., Harrell, R.A. and Handler, A.M. (2002) Germ-line transformation of the South American malaria vector, *Anopheles albimanus*, with a *piggyBac*/*EGFP* transposon vector is routine and highly efficient. *Insect Molecular Biology* 11, 291–297.

Perrimon, N., Ni, J.Q. and Perkins, L. (2010) In vivo RNAi: Today and tomorrow. *Cold Spring Harbor Perspectives in Biology* 2, a003640.

Peters, J.E., Makarova, K.S., Shmakov, S. and Koonin, E.V. (2017) Recruitment of CRISPR-Cas systems by *Tn7*-like transposons. *Proceedings of the National Academy of Sciences USA* 114, E7358–E7366.

Phelps, C.B. and Brand, A.H. (1998) Ectopic gene expression in *Drosophila* using GAL4 system. *Methods – a Companion to Methods in Enzymology* 14, 367–379.

Picardeau, M. (2010) Transposition of fly *mariner* elements into bacteria as a genetic tool for mutagenesis. *Genetica* 138, 551–558.

Pledger, D.W. and Coates, C.J. (2005) Mutant *Mos1 mariner* transposons are hyperactive in *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* 35, 1199–1207.

Poulton, B.C., Colman, F., Anthousi, A., Grigoraki, L., Adolfi, A. *et al.* (2021) Using the GAL4-UAS system for functional genetics in *Anopheles gambiae*. *JoVE (Journal of Visualized Experiments)* 170, e62131,

Prelich, G. (2012) Gene overexpression: Uses, mechanisms, and interpretation. *Genetics* 190, 841–854.

Qiao, H.H., Wang, F., Xu, R.G., Sun, J., Zhu, R.B. *et al.* (2018) An efficient and multiple target transgenic RNAi technique with low toxicity in *Drosophila*. *Nature Communications* 9(1), 4160.

Quinones-Coello, A.T., Petrella, L.N., Ayers, K., Melillo, A., Mazzalupo, S. *et al.* (2007) Exploring strategies for protein trapping in *Drosophila*. *Genetics* 175, 1089–1104.

Raphael, K.A., Shearman, D.C.A., Streamer, K., Morrow, J.L., Handler, A.M. and Frommer, M. (2011) Germ-line transformation of the Queensland fruit fly, *Bactrocera tryoni*, using a *piggyBac* vector in the presence of endogenous *piggyBac* elements. *Genetica* 139, 91–97.

Reznikoff, W.S. (2008) Transposon *Tn5*. *Annual Review of Genetics* 42, 269–286.

Riabinina, O. and Potter, C.J. (2016) The Q-system: a versatile expression system for Drosophila. In: Dahmann, C. (ed.) *Drosophila: Methods and Protocols,* 2nd edn, Vol. 1478. Humana Press Inc., Totowa, New Jersey, pp. 53–78.

Riabinina, O., Luginbuhl, D., Marr, E., Liu, S., Wu, M.N. *et al.* (2015) Improved and expanded Q-system reagents for genetic manipulations. *Nature Methods* 12, 219–222.

Rodrigues, F.G., Oliveira, S.B., Rocha, B.C. and Moreira, L.A. (2006) Germline transformation of *Aedes fluviatilis* (Diptera: Culicidae) with the *piggyBac* transposable element. *Memorias Do Instituto Oswaldo Cruz* 101, 755–757.

Rorth, P. (1996) A modular misexpression screen in Drosophila detecting tissue-specific phenotypes. *Proceedings of the National Academy of Sciences USA* 93, 12418–12422.

Rowan, K.H., Orsetti, J., Atkinson, P.W. and O'Brochta, D.A. (2004) *Tn5* as an insect gene vector. *Insect Biochemistry and Molecular Biology* 34, 695–705.

Rubin, G.M. and Spradling, A.C. (1982) Genetic transformation of *Drosophila* with transposable element vectors. *Science* 218, 348–353.

Ryder, E. and Russell, S. (2003) Transposable elements as tools for genomics and genetics in *Drosophila*. *Briefings in Functional Genomics and Proteomics* 2(1), 57–71. doi: 10.1093/bfgp/2.1.57

Sasakura, Y., Awazu, S., Chiba, S. and Satoh, N. (2003) Germ-line transgenesis of the *Tc1/mariner* super-family transposon *Minos* in *Ciona intestinalis*. *Proceedings of the National Academy of Sciences USA* 100, 7726–7730.

Scavarda, N.J. and Hartl, D.L. (1984) Interspecific DNA transformation in *Drosophila*. *Proceedings of the National Academy of Sciences USA* 81, 7515–7519.

Schetelig, M.F. and Handler, A.M. (2012) A transgenic embryonic sexing system for *Anastrepha suspensa* (Diptera: Tephritidae). *Insect Biochemistry and Molecular Biology* 42, 790–795.

Schetelig, M.F. and Handler, A.M. (2013) Germline transformation of the spotted wing drosophilid, *Drosophila suzukii,* with a *piggyBac* transposon vector. *Genetica* 141, 189–193.

Schetelig, M.F., Caceres, C., Zacharopoulou, A., Franz, G. and Wimmer, E.A. (2009) Conditional embryonic lethality to improve the sterile insect technique in *Ceratitis capitata* (Diptera: Tephritidae). *BMC Biology* 7, 4.

Schetelig, M.F., Gotschel, F., Viktorinova, I., Handler, A.M. and Wimmer, E.A. (2011) Recombination technologies for enhanced transgene stability in bioengineered insects. *Genetica* 139, 71–78.

Schinko, J.B., Weber, M., Viktorinova, I., Kiupakis, A., Averof, M. *et al.* (2010) Functionality of the GAL4/UAS system in *Tribolium* requires the use of endogenous core promoters. *BMC Developmental Biology* 10, Article 53.

Schinko, J.B., Hillebrand, K. and Bucher, G. (2012) Heat shock-mediated misexpression of genes in the beetle *Tribolium castaneum*. *Development Genes and Evolution* 222, 287–298.

Schulte, C., Theilenberg, E., Muller-Borg, M., Gempe, T. and Beye, M. (2014) Highly efficient integration and expression of *piggyBac*-derived cassettes in the honeybee (*Apis mellifera*) *Proceedings of the National Academy of Sciences USA* 111, 9003–9008.

Sentry, J.W., Yang, M.M. and Kaiser, K. (1993) Conditional cell ablation in *Drosophila*. *Bioessays* 15, 491–493.

Shelake, R.M., Pramanik, D. and Kim, J.Y. (2019) Evolution of plant mutagenesis tools: a shifting paradigm from random to targeted genome editing. *Plant Biotechnology Reports* 13, 423–445.

Shelton, A.M., Long, S.J., Walker, A.S., Bolton, M., Collins, H.L. *et al.* (2020) First field release of a genetically engineered, self-limiting agricultural pest insect: Evaluating its potential for future crop protection. *Frontiers in Bioengineering and Biotechnology* 7, 482.

Shi, H.F., Wormsley, S., Tschudi, C. and Ullu, E. (2002) Efficient transposition of preformed synaptic *Tn5* complexes in *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* 121, 141–144.

Singh, A. (1995) Enhancer trap technique – a novel tool for identification and developmental characterization of *Drosophila* genes. *Current Science* 68, 517–525.

Smith, D., Wohlgemuth, J., Calvi, B.R., Franklin, I. and Gelbart, W.M. (1993) *hobo* enhancer trapping mutagenesis in *Drosophila* reveals an insertion specificity different from *P* elements. *Genetics* 135, 1063–1076.

Spradling, A.C. and Rubin, G.M. (1982) Transposition of cloned *P* elements into *Drosophila* germ line chromosomes. *Science* 218, 341–347.

Stanford, W.L., Cohn, J.B. and Cordes, S.P. (2001) Gene-trap mutagenesis: Past, present and beyond. *Nature Reviews Genetics* 2, 756–768.

Stebbins, M.J., Urlinger, S., Byrne, G., Bello, B., Hillen, W. and Yin, J.C.P. (2001) Tetracycline-inducible systems for *Drosophila*. *Proceedings of the National Academy of Sciences USA* 8, 10775–10780.

Stern, D.L., Crocker, J., Ding, Y., Frankel, N., Kappes, G. *et al.* (2017) Genetic and transgenic reagents for *Drosophila simulan*s, *D. mauritiana, D. yakuba, D. santomea,* and *D. virilis*. *G3: Genes Genomes Genetics* 7, 1339–1347.

Stofanko, M., Kwon, S.Y. and Badenhorst, P. (2008) A misexpression screen to identify regulators of *Drosophila* larval hemocyte development. *Genetics* 180, 253–267.

Strecker, J., Ladha, A., Gardner, Z., Schmid-Burgk, J.L., Makarova, K.S. *et al.* (2019) RNA-guided DNA insertion with CRISPR-associated transposases. *Science* 365, 48.

Suganuma, R., Pelczar, P., Spetz, J.F., Hohn, B., Yanagimachi, R. and Moisyadi, S. (2005) *Tn5* transposase-mediated mouse transgenesis. *Biology of Reproduction* 73, 1157–1163.

Sumitani, M., Yamamoto, D.S., Oishi, K., Lee, J.M. and Hatakeyama, M. (2003) Germline transformation of the sawfly, *Athalia rosae* (Hymenoptera: Symphyta), mediated by a *piggyBac*-derived vector. *Insect Biochemistry and Molecular Biology* 33, 449–458.

Sundararajan, P., Atkinson, P.W. and O'Brochta, D.A. (1999) Transposable element interaction in insects: crossmobilization of *hobo* and *Hermes*. *Insect Molecular Biology* 8(3), 359–368. doi:10.1046/j.1365-2583.1999.83128.x

Tamura, T., Thibert, C., Royer, C., Kanda, T., Abraham, E. *et al.* (2000) Germline transformation of the silkworm *Bombyx mori* L. using a *piggyBac* transposon-derived vector. *Nature Biotechnology* 18(1), 81–84.

Tellier, M., Bouuaert, C.C. and Chalmers, R. (2015) *Mariner* and the *ITm* superfamily of transposons. *Microbiology Spectrum* 3(2), MDNA3-0033-2014.

Thibault, S.T., Singer, M.A., Miyazaki, W.Y., Milash, B., Dompe, N. *et al.* (2004) *P* and *piggyBac* transposons display a complementary insertion spectrum in *Drosophila*: A multifunctional toolkit to manipulate an insect genome. *Nature Genetics* 36, 283–287.

Thomas, J., Pritham Ellen, J., Chandler, M. and Craig, N. (2015) *Helitrons,* the eukaryotic rolling-circle transposable elements. *Microbiology Spectrum* 3, 3.4.03.

Trauner, J., Schinko, J., Lorenzen, M.D., Shippy, T.D., Wimmer, E.A. *et al.* (2009) Large-scale insertional mutagenesis of a coleopteran stored grain pest, the red flour beetle *Tribolium castaneum*, identifies embryonic lethal mutations and enhancer traps. *BMC Biology* 7, 73.

Uchino, K., Sezutsu, H., Imamura, M., Kobayashi, I., Tatematsu, K.I. *et al.* (2008) Construction of a *piggyBac*-based enhancer trap system for the analysis of gene function in silkworm *Bombyx mori*. *Insect Biochemistry and Molecular Biology* 38, 1165–1173.

Venken, K.J.T. and Bellen, H.J. (2005) Emerging technologies for gene manipulation in *Drosophila melanogaster*. *Nature Reviews Genetics* 6, 167–178.

Venken, K.J.T. and Bellen, H.J. (2007) Transgenesis upgrades for *Drosophila melanogaster*. *Development* 134, 3571–3584.

Venken, K.J.T., Schulze, K.L., Haelterman, N.A., Pan, H.L., He, Y.C. *et al.* (2011) *MiMIC*: A highly versatile transposon insertion resource for engineering *Drosophila melanogaster* genes. *Nature Methods* 8, 737–780.

Venken, K.J.T., Sarrion-Perdigones, A., Vandeventer, P.J., Abel, N.S., Christiansen, A.E. and Hoffman, K.L. (2016) Genome engineering: *Drosophila melanogaster* and beyond. *Developmental Biology* 5, 233–267.

Wang, H.G. and Fraser, M.J. (1993) TTAA serves as the target site for TFP3 lepidopteran transposon insertions in both nuclear polyhedrosis virus and *Trichoplusia ni* genomes. *Insect Molecular Biology* 1, 109–116.

Wang, H.H., Fraser, M.J. and Cary, L.C. (1989) Transposon mutagenesis of baculoviruses: analysis of TFP3 lepidopteran transposon insertions at the FP locus of nuclear polyhedrosis viruses. *Gene* 81, 97–108.

Wang, X.G., Ma, S.Y., Liu, Y., Lu, W., Sun, L. *et al.* (2019) Transcriptional repression of endogenous genes in BmE cells using CRISPRi system. *Insect Biochemistry and Molecular Biology* 111.

Warren, I.A., Fowler, K. and Smith, H. (2010) Germline transformation of the stalk-eyed fly, *Teleopsis dalmanni*. *BMC Molecular Biology* 11, 86.

Warren, W.D., Atkinson, P.W. and O'Brochta, D.A. (1994) The *Hermes* transposable element from the housefly, *Musca domestica*, is a short inverted repeat-type element of the *hobo*, *Ac*, and *Tam3* (*hAT*) element family. *Genetical Research* 64, 87–97.

Wen, W., Song, S.S., Han, Y.C., Chen, H.B., Liu, X.Z. and Qian, Q.J. (2020) An efficient screening system in yeast to select a hyperactive *piggyBac* transposase for mammalian applications. *International Journal of Molecular Sciences* 21, 13.

Wicker, T., Sabot, F., Hua-Van, A., Bennetzen, J.L., Capy, P. *et al.* (2007) A unified classification system for eukaryotic transposable elements. *Nature Reviews Genetics* 8, 973–982.

Williams, A.E., Sanchez-Vargas, I., Reid, W.R., Lin, J.Y., Franz, A.W.E. and Olson, K.E. (2020) The antiviral small-interfering RNA pathway induces Zika virus resistance in transgenic *Aedes aegypti*. *Viruses* 12, 18.

Xu, R.G., Wang, X., Shen, D., Sun, J., Qiao, H.H. *et al.* (2019) Perspectives on gene expression regulation techniques in Drosophila. *Journal of Genetics and Genomics* 46, 213–220.

Yant, S.R., Huang, Y., Akache, B. and Kay, M.A. (2007) Site-directed transposon integration in human cells. *Nucleic Acids Research* 35.

Yonemura, N., Tamura, T., Uchino, K., Kobayashi, I., Tatematsu, K. *et al.* (2012) PhiC31 integrase-mediated cassette exchange in silkworm embryos. *Molecular Genetics and Genomics* 287, 731–739.

Yuen, J.L., Read, S.A., Brubacher, J.L., Singh, A.D. and Whyard, S. (2008) Biolistics for high-throughput transformation and RNA interference in *Drosophila melanogaster*. *Fly* 2, 8.

Yusa, K. (2015) *piggyBac* transposony. In: Craig, N.L., Chandler, M., Gellert, M. *et al.* (eds) *Mobile DNA III.* ASM Press, Washington, DC, pp. 873–890.

Yusa, K., Zhou, L.Q., Li, M.A., Bradley, A. and Craig, N.L. (2011) A hyperactive *piggyBac* transposase for mammalian applications. *Proceedings of the National Academy of Sciences USA* 108, 1531–1536.

Zhang, D.S., Zhou, W.G., Yin, C., Chen, W.T., Ozawa, R. *et al.* (2006) Misexpression screen for genes altering the olfactory map in *Drosophila*. *Genesis* 44, 189–201.

Zimowska, G.J. and Handler, A.M. (2006) Highly conserved *piggyBac* elements in noctuid species of Lepidoptera. *Insect Biochemistry and Molecular Biology* 36, 421–428.

2 Inducible and Repressible Systems for Transgene Expression

Marc F. Schetelig[1]*, Rosemary S. Lees[2], Rocco D'Amato[3] and Mark Q. Benedict[4]

[1]*Justus-Liebig-University Gießen, Department of Insect Biotechnology in Plant Protection, Gießen, Germany;* [2]*Department of Vector Biology, Liverpool School of Tropical Medicine, Liverpool, United Kingdom;* [3]*Polo GGB - Polo d'Innovazione di Genomica, Genetica e Biologia, Terni Italy;* [4]*Centers for Disease Control and Prevention (CDC), Div. Parasitic Diseases and Malaria, Atlanta, GA USA*

2.1 Introduction

In the context of applications of transgenic insects, the capability to control gene expression enables genetic circuitries as well as unique expression systems to be evaluated and engineered. Close regulation of expression likewise enables the use of transgenes whose constitutive expression, even when limited to a particular tissue and stage, would be harmful, lethal, or cause phenotypic effects that would not be compatible with creating, maintaining, or deploying transgenic insects. Therefore, identifying and adapting existing inducible or repressible gene expression systems is critical for creating a suite of flexible tools for transgenic insects. For example, the ability to induce or repress transgene expression could be used to achieve male sterility in insects for population reduction or for specific functions such as female-killing as a sex separation method to allow a male-only release. Ultimately, the requirement for gene expression systems that can be controlled by an easily applied external treatment to facilitate the production of transgenic animals for release

or other production settings is fundamental to the economy and success of such efforts.

There is a variety of inducible or repressible gene expression systems available that merit further testing in insects. They vary from adaptations of specific regulatory machinery for exclusive use in the species of origin, transfer to another species of interest when gene regulation is conserved between species, to more complex engineered systems, such as inducible homo-dimerization and the Q-system. All these systems have to face the possibility of the basal level of expression or leakiness, particularly important when dealing with lethal genes. Moreover, small molecule regulators could have an impact on insect fitness; for example, antibiotics could disrupt the microbiome or impact the mitochondrial function.

In this chapter, we provide an overview of inducible and repressible gene expression systems with a specific focus on those that have been demonstrated in insects or which, in our estimation, merit consideration for further development. We do not discuss bipartite systems such as Gal4/UAS except when controlling their expression could be

---

*Corresponding author, email: marc.schetelig@agrar.uni-giessen.de

achieved other than by crossing lines. We also do not discuss systems that harbour blood-feeding-induced regulatory regions of genes, as these can typically only be used in specific insects or sexes. Instead, we focus on versatile systems where some externally applied conditions can control either up- or downregulation of gene expression, and the removal returns expression to the previous state.

2.2   Naturally Occurring Systems of Conditional Expression

2.2.1   Heat shock – *hsp70*

The development of one of the early candidates for inducible gene expression was stimulated by observations of an impressive increase in gene transcription and translation in *Drosophila melanogaster* when exposed to near-lethal heat shocks, initially observed as puffing of the polytene chromosomes in the vicinity of various heat shock genes (Tissieres *et al*., 1974). The puffing, and concomitant increase in transcription, was rapid, inducible, reversible and correlated with significantly increased expression of a highly conserved family of large protein-encoding genes (Lindquist, 1986). Heat shock genes are both transcriptionally (Morimoto, 1993) and translationally regulated, with their translation being favoured over other mRNAs during heat shock (Storti *et al*., 1980). Their role is that of chaperonins, which protect proteins that otherwise might be damaged during heat stress (Parsell and Lindquist, 1993; Saibil, 2013). However, they have numerous other functions and figure prominently as biomarkers of different stresses and several pathologies. The heat shock suite of genes is ubiquitous and, as discussed below, many of the transcriptional control elements are sufficiently conserved to allow faithful interspecific expression of transgenes.

A *Drosophila* heat shock gene, *hsp70*, became a popular choice for transgene expression due to its high induction above background – at least two orders of magnitude – and low constitutive expression (Lindquist, 1986). Deletion analysis of *hsp70* promoters identified the essential portions of the promoters for heat induction (Pelham, 1982) containing one or more conserved 'heat shock elements' (Bienz and Pelham, 1987; Sakurai and Enoki, 2010). These consist of a palindrome GAAnnTTCnn that often occurs in tandem arrays, usually located within a few hundred bases of the TATA box. These promoters were particularly interesting since the transcription factors necessary for induction were functionally conserved in *Xenopus* (Voellmy and Rungger, 1982), green monkey (Amin *et al*., 1987) and tobacco (Wing *et al*., 1989). Additional heat-shock-specific expression is determined by 5′ untranslated regions (UTRs), which determine preferential translation of the transcripts during heat shock in humans (Vivinus *et al*., 2001) and *Drosophila* (Klemenz *et al*., 1985; Hess and Duncan, 1996). Other sequences determining stress-specific transcript regulatory mechanisms were eventually determined to reside in the 3′ UTR, promoting accelerated *hsp70* transcript degradation when not under heat shock (Lindquist and Petersen, 1990). *Drosophila melanogaster*, *Ceratitis capitata* and the blowfly *Lucilia sericata* (Meigen) *hsp70* promoters quickly found a use for transgene expression (Tachibana *et al*., 2005; Kalosaka *et al*., 2009), though often without using the entire set of promoter, 5′ and 3′ UTR sequences that were eventually identified as being crucial for low constitutive expression, high inducibility and preferential translation (Papadimitriou *et al*., 1998; Kalosaka *et al*., 2006).

The seminal observation of chromosome heat shock puffs was recapitulated at the loci of transgene insertions containing *P* element insertions of *hsp70* 5′ regulatory sequences (+194 bp upstream of the transcription start site) fused to a β-galactosidase reporter (Lis *et al*., 1983). Upon heat shock, high levels of β-galactosidase expression were observed with no detectable background expression. These results indicated that only 5′ sequences were necessary for authentic expression. In the pre-PCR era, where sequencing insertions from libraries was laborious, unusual puffing provided

an easily observed indicator of the transgene location.

Many efforts to use the *hsp70* promoter revealed significant constitutive expression in *Drosophila* (Steller and Pirrotta, 1985a,b) and the first germline transformation of a malaria mosquito, *Anopheles gambiae* (Miller *et al.*, 1987). This expression led to significant non-shock expression, or leakiness, in systems designed to achieve inducible expression using the *hsp70* promoter. Discrimination was greatly enhanced once the correct temperature to induce shocks was determined (Sakai and Miller, 1992). This should be kept in mind when testing the expression of *hsp70*-driven transgenes: the maximal temperature for heat shock gene induction is species-specific and dependent upon the culture temperature. However, it can easily be determined by performing survival analysis (Benedict *et al.*, 1991; Patil *et al.*, 1996).

The *D. melanogaster hsp70* promoter and the 5′ UTR (approximately 400 bp and 200 bp, respectively) were also used to express *alcohol dehydrogenase* (*adh*), which was expected to create inducible tolerance to ethanol but whose expression can also be counter-selected with 1-pentyne-3-ol (O'Donnell *et al.*, 1975; Bonner *et al.*, 1984). Shocked flies demonstrated increased tolerance to usually lethal concentrations of ethanol. While the purpose of these studies was to analyse mutations affecting the heat shock response, a useful selectable and inducible marker was created.

Numerous heat shock genes have been identified (Zhao and Jones, 2012), and both *Drosophila* and native heat shock promoters have been used for inducible transgene expression. With medfly (*Ceratitis capitata*), the native *hsp70* promoter was tested in comparison with a *D. melanogaster hsp70* promoter to drive *lacZ* expression in 15 independent lines (Kalosaka *et al.*, 2006), using the SV40 terminator, but not the native *hsp70* 3′ UTR. Despite superficial 5′ regulatory sequence conservation, the *Drosophila* promoter generally had low inducible expression relative to the *C. capitata* promoter. Both promoters resulted in low levels of constitutive expression, which might be acceptable in certain applications. The authors

suggest that further suppression of constitutive expression could have been obtained if the 3′ UTR had been used. Regardless, both promoters resulted in expression below that of the native heat shock genes.

In the yellow fever mosquito, *Aedes aegypti*, six members of the *hsp70* family have been identified (Gross *et al.*, 2009) and tested to find a native promoter capable of providing low constitutive expression and high inducibility of a luciferase gene (Carpenetti *et al.*, 2012). Two regions containing the promoter and 5′ UTR sequences, extending up to −1456 bp, drove transcriptional activation and an approximately tenfold increase in reporter expression relative to uninduced mosquitoes after a 1 h treatment at 39°C, and 25- to 50-fold increase in expression at 4 h post-treatment. In the absence of heat shock, there was evidence of low levels of luciferase activity.

The functionality of the *D. melanogaster hsp70* promoter control sequences across various animal species has further been demonstrated in transgenic *Bombyx mori*. Inducible expression of a *B. mori* nuclear receptor, *Ftz-F1*, was obtained using both the 5′ and 3′ sequences of a *D. melanogaster hsp70* gene (Uhlirova *et al.*, 2002). While expression was again detected in non-induced moths, high levels of transient induction were obtained. Moreover, the *hsp70* promoter has been used to expresses double-stranded RNA from a hairpin construct to trigger RNAi-mediated knockdown of mRNA of the *B. mori* eclosion hormone (Dai *et al.*, 2007). This study used only *D. melanogaster hsp70* promoter sequences to drive the transgene. Generally, RNAi might be an application in which low levels of constitutive expression are acceptable. Another study established the so-called *Pogostick* heat-inducible expression vectors using the *D. melanogaster hsp70* promoter and 3′ UTR sequences to overexpress or downregulate genes of interest in species that can be transformed with vectors based on the *piggyBac* transposable element (Chen *et al.*, 2011). The system proved functional in *D. melanogaster* and the butterfly *Bicyclus anynana*. With the rise of CRISPR technologies for genome editing, the *hsp70* promoter and its 3′ UTR were also used to

establish transgenic, inducible Cas9 expression lines in *Drosophila suzukii* with variable amounts of transient Cas9 expression (Yan *et al.*, 2021). After heat shock, the established lines showed up to 85.7% gene editing efficiency in the germline demonstrated by creating knockout mutations in the *yellow* gene of *D. suzukii*.

These examples provide evidence of the broad use of *hsp70* promoters; however, despite potentially high inducibility, basal expression from the *hsp70* promoter should be considered, particularly when the transgene to be induced might be toxic or when low expression is otherwise unacceptable. The safest approach for creating such constructs is to include a native promoter, 5′ and 3′ UTR sequences in transgene fusions to maximize the chances of achieving highly inducible expression and low levels of leakiness. As an alternative, the CRISPR/Cas9 system could be used to target a genomic region containing an *hsp* gene cluster to provide the optimal epigenetic context for heat-inducible expression (see Ahmed and Wimmer, Chapter 5, this volume).

2.2.2   Natural temperature-sensitive lethal elements and mutations

One application for temperature-controlled systems like the *hsp70* promoters and other temperature-sensitive elements can be conferring lethality in insect pest control programmes like the sterile insect technique (SIT) (see Scott *et al.*, Chapter 17, this volume). Classical genetic sexing strains (GSS) for SIT programmes in *C. capitata* use elevated temperature during embryogenesis to achieve sex separation (Franz, 2005). The GSS females are homozygous for a *temperature-sensitive lethal* (*tsl*) mutation, while males have an additional wild-type (WT) rescue *tsl* allele translocated on the Y chromosome. Thus, incubation of embryos at 33–36°C causes female-specific lethality while males survive the treatment (Robinson, 2002; Franz *et al.*, 2021). Since GSS have been successfully used to produce billions of sterile male insects for field release,

it is attractive to consider other applications of natural temperature-sensitive elements to interfere with and control the expression of essential genes for viability or fertility.

A *C. capitata tsl* mutation remains to be identified but would likely be a mutation in an essential gene that encodes a protein that is unstable at elevated temperatures. The first example of a dominant temperature-sensitive (DTS) lethal mutation explored to control and confer lethality under certain environmental conditions was the *β2 proteasome subunit* gene *(Prosβ2′),* a proteasome protein of *D. melanogaster* (Smyth and Belote, 1999). Prosβ2′ caused pupal lethality at 29°C, but allowed survival to adulthood at 25°C when tested in the tephritid *Anastrepha suspensa* (Nirmala *et al.*, 2009). Other temperature-dependent mutations of the conserved sex determination gene *transformer 2*, essential for female development, were first studied in *D. melanogaster,* and mutant Tra2 proteins functioned normally at 16°C but led to multiple sex determination-related effects at 29°C (Belote and Baker, 1982). Later, *D. suzukii* and *C. capitata* strains, carrying temperature-sensitive mutations in conserved amino acids of the *tra2* gene, were generated by CRISPR/Cas9 HDR technology (Li and Handler, 2017; Aumann *et al.*, 2020). While the results were promising, the full potential for applications could not be evaluated, due to the low survival of *D. suzukii* above 26°C and the inability to find a permissive temperature compatible with regular breeding for *C. capitata* strains. In addition, temperature-sensitive mutations in the dynamin GTPase *shibire* gene were discovered in *D. melanogaster* (Grigliatti *et al.*, 1973; Chen *et al.*, 1991; van der Bliek and Meyerowitz, 1991), resulting in lethal or paralytic phenotypes. Transferring one of the mutations, *shi*[ts1], via CRISPR/Cas9 mutagenesis into the tephritid *Bactrocera tryoni* resulted in lethality in some individuals even at the 21°C rearing temperature, which was a more severe phenotype than in *D. melanogaster*, and therefore the generated strains were not suitable for further evaluation (Choo *et al.*, 2020). With the advent of CRISPR/Cas9 technology (see Concha and Papa, Chapter 7, this volume), it has become

technically possible to make targeted mutations that could make the encoded protein temperature-sensitive.

Another temperature-sensitive system is represented by inteins (intervening proteins), described as 'protein introns', able to excise themselves from a polypeptide and join the N- and C-terminal flanking sequences to create a WT version of the encoded protein. Temperature-sensitive Gal4 molecules have been generated using conditionally splicing inteins. The capacity of the mutant inteins to be spliced out of the host protein was shown to be sufficient to abolish all Gal4 activity, both in S2 cell lines and in transgenic *Drosophila* (Zeidler *et al.*, 2004). Moreover, in *Drosophila*, splicing of a temperature-sensitive intein (TS-ClvR^dbe) was able to rescue the activity of an essential gene, *dribble*, for conditional gene drive (Oberhofer *et al.*, 2021).

All temperature-sensitive systems have in common that they would be optimal if regulated by external heat stimuli whilst harbouring minimal genomic modifications of natural genes. In truth, those lines carry a much lower level of genomic mutations than classical genetic strains generated via chemosterilization or radiation. Such systems are promising technologies because heat could induce gene expression in many developmental stages, as long as the effect is viable and controllable under regular rearing conditions of the insect.

2.2.3 Glucose repression

The expression of the *Drosophila* α-amylase gene can be highly repressed by supplying sugars in *Drosophila* diets (Benkel and Hickey, 1986). Repression of more than 100-fold can be accomplished by the simple addition of dextrose in the diet (Benkel and Hickey, 1987). In contrast to *hsp70*-driven gene expression, only 5′ promoter sequences (478 bp) are necessary for repression in *Drosophila,* and the same also function in heterologous yeast expression experiments (Hickey *et al.*, 1994), showing that the *cis*-acting elements controlling transcription are conserved. We are not aware of any reports of their use in non-drosophilid insects, though it seems likely that regulation of this important enzyme would be conserved.

2.2.4 Metallothionein

The environmental availability of complex carbohydrates requires an animal to modulate expression of the *α-amylase* gene. Conversely, the environment presents threats to which the organism must respond, requiring induction of other systems, such as the *hsp70* regulatory sequences evolving as a response to heat stress and other genes as protection against environmental toxins. One mechanism of increased metal tolerance in *D. melanogaster* is naturally occurring duplications of metallothionein genes (*Mtn*) (Maroni *et al.*, 1987). Metallothioneins (MTs) are small, conserved proteins that bind and sequester ions of copper, zinc and cadmium in fungi (yeast), plants and animals (Coyle *et al.*, 2002). They lack aromatic amino acids but are cysteine-rich motifs that are necessary to bind and sequester metal ions.

Not surprisingly, tolerance to environmental threats might be under greater selection pressure in some environments than in others. While the inter-population variation in *D. melanogaster* tolerance to heavy metals is determined partly by duplication of *Mtn* genes, individual gene expression can be modulated in response to heavy metal exposure. Increased synthesis of MTs occurs via transcriptional and translational regulation. *Mtn* transcription increases in response to heavy metals are widely observed in mammalian cells and animals (Zhu and Thiele, 1996).

To investigate the sequences responsible for metal regulation of *Mtn* in *D. melanogaster*, various portions of the *Mtn* gene have been introduced into flies by P-element-mediated germline transformation (Otto *et al.*, 1987). Metal-regulated *Mtn* expression required sequences between –373 and +54 bp of the transcription start site. *Mtn* promoters contain conserved metal response elements (MRE) that are necessary for transcriptional

induction. An MRE consists of a 12 bp DNA segment repeated within the promoter, which functions as a metal ion-dependent enhancer (Andersen *et al.*, 1987). The *Mtn* promoter of *Drosophila* increased accumulation of transgene mRNA when induced by metals in baby hamster kidney cells. This indicated that, as in glucose repression of *α-amylase*, the *cis*-acting sequences and *trans*-acting factors responsible for metal regulation are highly conserved.

Induction of a *D. melanogaster Mtn* MT-1 promoter has been accomplished in mosquito cells (*Aedes albopictus*) transformed to express *Escherichia coli* β-galactosidase (Kovach *et al.*, 1992). Copper and, to a lesser extent, cadmium were both inducers. The relative magnitude of induction by copper was 10- to 20-fold over the non-induced state. The cell line studied contained approximately 60 copies of the transgene, therefore interpreting these results in the context of transgenic *Ae. albopictus* carrying only one transgene is difficult and, to our knowledge, has not been attempted.

2.2.5   *lac* inducible systems

The *lac* operon of *E. coli* is a multi-gene system repressed by the presence of lactose. In *E. coli*, *lac* structural genes are transcriptionally repressed by the *lac* repressor (*LacI*, termed *LacR* by some authors), which, when not bound to an inducing chemical, binds to the lac operator (*lacO*) sequence located between the promoter and the regulated gene. Thus, in the absence of an inducer, transcription is blocked. Various inducers, including β-galactosides, can bind to and block *LacI*, allowing transcription. To prevent loss of the inducer via metabolism, a chemical reagent such as isopropyl-β-D-thiogalactopyranoside (IPTG) is often used. Adding a β-galactoside such as lactose or IPTG causes the repressor to undergo a conformational change, allowing RNA polymerase to access the promoter and initiate transcription.

While the *lac* operator–repressor interaction is a prokaryotic system, it has been utilized for inducible protein expression in *Drosophila* cells (Wakiyama *et al.*, 2011), mice (Wu *et al.*, 1997; Cronin *et al.*, 2001) and canine cells (Lin *et al.*, 1997), and for reporter gene expression in *Borrelia burgdorferi* (Blevins *et al.*, 2007). All components required for regulation of gene expression by *lac* can be included in a single transformation vector. This system has not been developed in mosquitoes and was only used in S2 cells of *D. melanogaster* (Wakiyama *et al.*, 2011). More than 25-fold induction of transgene expression was achieved in transient assays, and the *lac* operator–repressor system was observed to function in transformed cell lines. Transfection of a vector containing a DNA sequence encoding the *lac* repressor protein driven by a modified *Actin5C* promoter could regulate a gene stably integrated into the S2 chromosome. Repression was most effective if the operator sequence was placed between the TATA box and the cap site, suggesting that *lacR* bound to the operator could be blocking transcription initiation.

A heterologous inducible *lacI* 'P_{tac}' system has also been developed, including the hybrid *trp/lac* promoter P_{tac} (de Boer *et al.*, 1983) and the *lac* repressor from *E. coli*. This inducible system has been used for gene expression analysis in a range of bacteria, for example *Pseudomonas* (de Lorenzo *et al.*, 1993) and *Myxococcus xanthus* (Letouvet-Pawlak *et al.*, 1990), and for induction of expression using lactose (Khlebnikov and Keasling, 2002) and temperature (Xue *et al.*, 1996) in *E. coli*. Variants have been developed that are thermosensitive and others that are cold-responsive (Makrides, 1996), so the system has the potential to be very flexible in its application.

Previous attempts to apply the technique in human systems have failed due to the toxicity of IPTG (Makrides, 1996). Still, reversible regulation of gene expression has been achieved in mouse embryonic stem cells, giving a five- to tenfold IPTG induction of transgene expression (Caron *et al.*, 2005). Additionally, a transgenic inducible *lac* system has been developed *in vivo* in axolotls to investigate regeneration gene function (Whited *et al.*, 2012), and IPTG-mediated gene silencing has been demonstrated in mice cells (Grespi *et al.*, 2011). *lacI* expression can be

placed under the control of other promoters, as demonstrated by the production of conditional mutants in *Helicobacter pylori* (Boneca *et al*., 2008), suggesting the potential for reducing toxicity and achieving a broader application. The *lac* system has even been combined with the Tet system (see below) and named the *lac*/Tet dual-inducible system for induction of two genes simultaneously in mammalian cell lines by adding IPTG and Tet, which were not cytotoxic when added at optimal concentrations (Liu *et al*., 1998).

2.3   Synthetic Systems

2.3.1   Tetracycline-mediated expression

The tetracycline-based binary expression systems (Tet systems) are among the best characterized and versatile inducible expression systems originating from *E. coli* (Gossen and Bujard, 1992). There are two variants of the Tet system: one is the tTA system ('Tet-Off' system) and the other the rtTA system ('Tet-On' system). These are discussed in more detail in various contexts in Chapters 17, 21 and 23 (this volume). The system consists of two parts, the first being the driver construct with tetracycline-controlled transactivator (*tTA*) produced under the control of a promoter that usually provides tissue- or cell-specificity. The second part is the effector, which contains a target gene under the control of a minimal promoter sequence, which thus has a naturally low level of activity. Expression from a minimal promoter can be increased in the presence of an enhancer or by a positive feedback system. In the most commonly used example developed by Gossen and colleagues in mammalian cells (Gossen and Bujard, 1992; Gossen *et al*., 1995), the human cytomegalovirus promoter (*Pcmv*) is combined with Tet operator sequences (*tetO*). In the absence of tetracycline (or the derivative semi-synthetic antibiotic doxycycline), tTA binds to *tetO* and activates the *Pcmv* promoter to initiate the transcription of an effector gene. In the presence of tetracycline, tTA undergoes a

conformational change and dissociates from *tetO*, terminating transcription of the target gene. In the Tet-On system, the driver construct has a mutant Tet repressor (rtTA), while the effector construct is the same as found in the Tet-Off system. In this case, the system works through an opposite mechanism. In the absence of tetracycline, rtTA does not bind to *tetO* sequences, and the target gene is transcriptionally inactive. In the presence of tetracycline, rtTA binds to *tetO*, and *Pcmv* activates transcription of the target gene. Tet systems have been demonstrated in animal models, including mice and rats (Lewandoski, 2001; Zhu *et al*., 2002). However, problems related to the system include leaky expression caused by promoter-dependent or integration site-dependent effects. These effects can compromise the stringent regulation of transgene expression.

In insects, Tet-mediated expression has been used in *D. melanogaster* to establish conditional lethal systems. The conditional female-lethal systems were initially based on female-specific yolk protein promoters expressing tTA and driving lethal effectors (Heinrich and Scott, 2000; Thomas *et al*., 2000). The technology was referred to as the 'release of insects carrying a dominant lethal', RIDL (Thomas *et al*., 2000). In contrast to the sterile insect technique that leads to population suppression by releasing biologically sterile insects, RIDL and variants thereof are described as the release of fertile adults that confer unisex or female-specific lethality in developmental stages of their offspring as described above. Subsequently, enhancer/promoters of genes that are expressed during the blastoderm stage, like *serendipity α* (*sry α*) (Schweisguth *et al*., 1990), have been used to limit the effect of the transgene to the embryonic stage (Horn and Wimmer, 2003). In *D. melanogaster*, the *hid* gene driven by tTA under the control of a cellularization gene promoter produced embryonic lethality (Horn and Wimmer, 2003). Both RIDL and transgenic embryonic lethality systems based on the Tet-Off system were then transferred to different agriculturally, veterinary and medically relevant insect pest species.

In mosquitoes, the first Tet-system transgenics were established in *Ae. aegypti* using a construct containing a tetracycline-repressible transcriptional activator (tTAV), under control of TetO, and a minimal promoter from Drosophila *hsp70* (Phuc *et al.*, 2007). Then, a second version was created using the promoter from the *Ae. aegypti Actin-4* (*AeAct-4*) gene that drives the expression of tTA in a stage-, tissue- and sex-specific manner (Fu *et al.*, 2010). *AeAct-4* is expressed in the indirect flight muscles (IFM) of female pupae. The *AeAct-4* regulatory sequence was used to drive tTA expression in a driver line and crossed to a transgenic effector line carrying *DsRed* under the control of a tetracycline responsive element (tRE). The expression of DsRed was fully repressed by adding tetracycline. Crossing the strain carrying the *AeAct-4-tTA* construct to transgenic lines containing lethal effector genes *Nipp1Dm* and *Michelob_x* under tRE control produced a flightless phenotype in female progeny, with both constructs due to the expression of the lethal effector genes in the IFM. A further driver construct was made using the native *AeAct-4* sequence, which produces sex-specific alternative splicing, to prevent the expression of functional tTA protein in males, even if the promoter is active. In this case, the transgenic line obtained, OX3604C, when reared in the absence of tetracycline, produced a highly penetrant female-specific flightless phenotype. In contrast, in the presence of tetracycline, only 0.3% of females showed the flightless phenotype.

The Tet system has also been shown to be capable of controlling gene expression in *Anopheles stephensi*. In this case, a promoter sequence from the *An. gambiae SRPN10* gene was used to drive the production of Tet-dependent transactivators (Lycett *et al.*, 2004). A driver construct was developed for Tet-Off and Tet-On systems along with an effector line, which carries a *lacZ* reporter gene regulated by a *tetO* sequence. The progeny of crosses between the driver and effector lines expressed β-galactosidase in a significant fraction of haemocytes and pericardial cells for both Tet systems. Tet systems could be useful to characterize the function of many genes involved in mosquito development, innate immunity and parasite transmission. Generally, this system could improve the sterile insect technique (SIT) for mosquito control, particularly when the effectors make stock maintenance difficult or impossible.

Tet systems for the purpose of insect pest control have also been established in many agriculturally important and livestock pest insects, such as the Mediterranean fruit fly *C. capitata* (Schetelig *et al.*, 2009; Ogaugwu *et al.*, 2013), the Caribbean fruit fly *Anastrepha suspensa* (Loew) (Schetelig and Handler, 2012a,b), the pink bollworm *Pectinophora gossypiella* (Saunders) (Morrison *et al.*, 2012), the Australian sheep blowfly *Lucilia cuprina* Wiedemann (Yan and Scott, 2015), the New World screwworm *Cochliomyia hominivorax* (Diptera: Calliphoridae) (Concha *et al.*, 2016), the Mexican fruit fly *Anastrepha ludens* (Loew) (Diptera: Tephritidae) (Schetelig *et al.*, 2016), and recently in the cherry vinegar fly *D. suzukii* (Schetelig *et al.*, 2021). Successful in laboratory, semi-field cage and small-scale field tests, transgenic approaches for population suppression have reached the stage of pilot control trials on Grand Cayman (Harris *et al.*, 2012) and in Brazil (Carvalho *et al.*, 2015).

Following the tTA paradigm, several small-molecule regulated systems have been developed which follow a similar design, with a bacterial DNA-binding domain fused to the VP16 transcription activation domain. Erythromycin-Off, Biotin-On, Vanillic acid regulated, Phloretin-Off, Bile acid-Off, and Quinic acid systems have been developed for expression control (Jaffri *et al.*, 2020), though they have not yet been widely used in transgenic insects. Indeed, a recent attempt to use corresponding small-molecule ligands to control repressors from the p-CymR operon from *Pseudomonas putida*, PipR operon from *Streptomyces coelicolor*, TtgR operon from *P. putida* and the VanR operon from *Caulobacter crescentus* failed to demonstrate a concentration-dependent decrease in marker expression in *D. melanogaster*, unlike the parallel experiment with tTA flies (Gamez *et al.*, 2021).

2.3.2 Dimerization

The use of dimerization-dependent inducible gene expression is a further example of engineering inducible or repressible transgene expression using an endogenous regulatory system as a starting point. Interactions between pairs of proteins facilitate molecular signalling by altering proximity and orientation of proteins, create temporal and spatial boundaries, enhance reaction specificity and regulate gene expression (Klemm *et al.*, 1998). The production of dimers from monomers may be stable or dynamic. Interaction by dimerizing two signalling proteins can be induced with high affinity and specificity by an organic molecule with two binding motifs, a dimerizer, which triggers many cellular processes. If the motifs are identical, two identical molecules are joined, known as homodimerization, and where two different molecules are bound and induced to interact, it is known as heterodimerization.

Synthetic dimerization can be used to investigate molecular pathways by activating or inhibiting them (Klemm *et al.*, 1998). For example, dimerization proteins can be created that lack functional domains and so form ineffective dimers, so-called 'dominant negatives', whose inhibition is dose-dependent, or synthetic ligands can be applied that induce artificial interaction between two proteins by bringing them into close proximity (Spencer *et al.*, 1993). This latter reaction is reversible by the addition of a second competing ligand with only one binding site.

It is also possible to make protein–protein interactions dimerizer-inducible by fusing proteins to recognized binding motif(s). The use of dimerizers, bivalent chemical inducers of dimerization, allows regulation of gene expression by inducing proximity between the DNA-binding and activation domains of transcription factors, expressed as two fusion proteins with ligand-binding domains (Pollock and Clackson, 2002). In this way, adding or removing a dimerizer leads to transcriptional activation of a gene of interest downstream of a promoter containing a binding site for the DNA-binding domain. If the ligand-binding domains are

unreactive in the absence of the ligand and correlated promoter, transcription of the gene of interest only occurs upon the addition of the dimerizer. Tight regulation can be achieved, and the modularity allows stepwise optimization of a system. The ability to fuse any proteins to the binding domain/s makes the system very adaptable. The effectiveness of this system is greater if a heterodimerizer is used or if proteins from the target organism can be employed.

Protein interactions with nucleotide-binding and oligomerization domain-like (NOD) receptors (NLRs) in *Hydra magnipapillata*, which are essential for the innate immune system, were identified through the co-transfection of chimeric hydra sequences fused to FK506 binding protein (FKBP) into human embryonic kidney cells and the addition of a commercially available cell-permeable homodimerization ligand. Concentration-dependent protein binding was induced by dimerization of FKBP (Lange *et al.*, 2011). Immuno-precipitation products were collected, and co-precipitation signalled that endogenous interaction may occur with the target NLR; high levels of co-precipitation were achieved upon the addition of 100 nM AP20187 dimerizer to proteins in which, in its absence, only a weak interaction was detectable.

AP20187 dimerizer-induced apoptosis of macrophages has been achieved in transgenic mice through the dimerizer-activated expression of the *FKBP–Fas* suicide gene (Burnett *et al.*, 2004). Macrophage-like cells were reduced by 78.3% in *in vitro* cultures treated with dimerizer, with no impact on viability seen in treated wild-type cells. An IC50 of 0.37 nM was demonstrated for the dimerizer. Daily peritoneal injections of AP20187 at 10 mg/kg caused observable depletion of macrophages within 24 h, and a decrease of nearly 90% was seen after five days of treatment. Mice injected with the dimerizer, which thus had reduced macrophage levels, had reduced capacity to clear injected *Yersinia pestis* bacteria. Some reversibility was seen after seven days without treatment.

The iDimerize-regulated transcription system is a form of inducible dimerization (Fig. 2.1). Proteins of interest are fused to
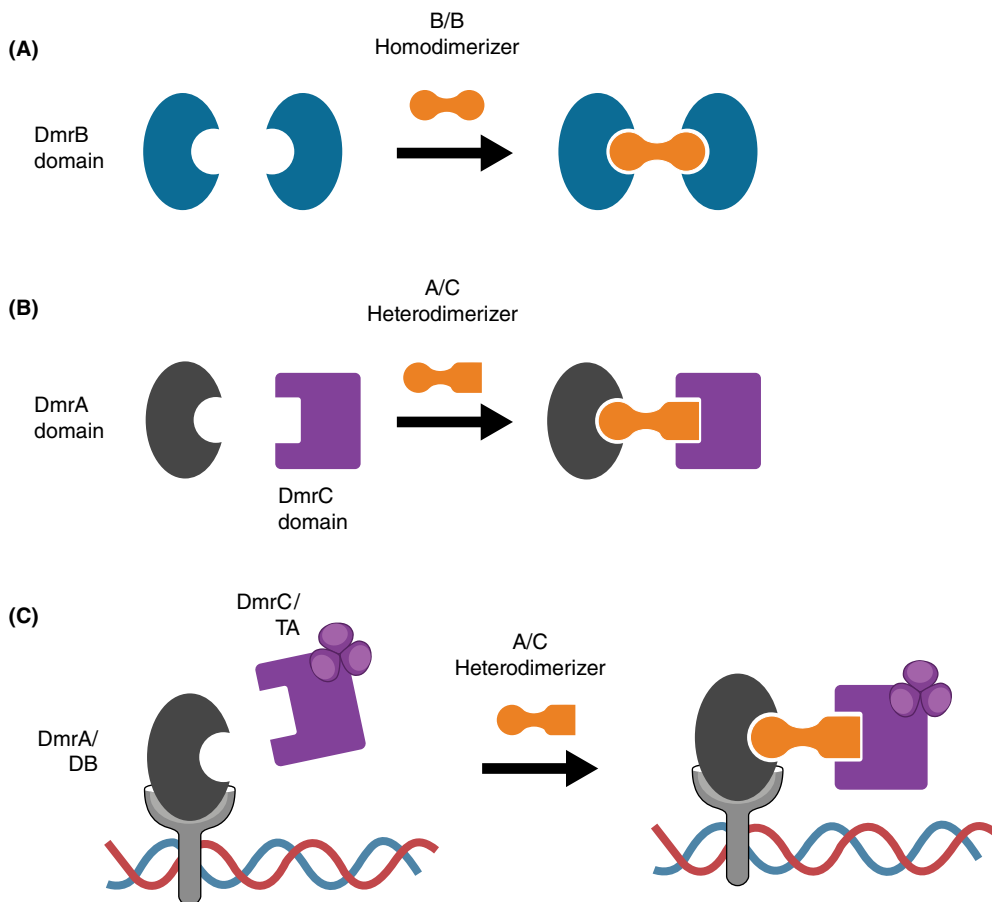
Fig. 2.1. The iDimerize inducible dimerization system. (A) The inducible homodimerization system also requires a synthetic, cell-permeable ligand for induction. The B/B homodimerizer is used to induce homodimerization of fusion proteins containing the DmrB domain inducing the transcription of the gene of interest. **(B)** The DNA-binding domain, DmrA, recognizes and binds sequences within the inducible promoter. Transcription of the gene of interest occurs when the DmrA and a transcription activation domain, DmrC, both bind to the synthetic, cell-permeable ligand which is applied to induce transcription. **(C)** The heterodimerization system motifs, fused to a transcription activation domain (TA) and a DNA binding domain (DB), respectively, are bound by the heterodimerizer (A/C) and induce transcription of the gene of interest.

domains recognized by a synthetic, bivalent, cell-permeable dimerizer, which may be an A/C heterodimerizer or a B/B homodimerizer, depending on whether the proteins targeted by dimerization are similar or different. When the dimerizer is added, the two binding ligands can interact, resulting in an interaction of the proteins of interest in a dose-dependent manner. Transcription activation of a target gene can be controlled in this manner by inserting an inducible promoter upstream that is recognized by a DNA-binding component that binds to a transcription activation component only in the presence of a heterodimer. Dose-responsive transcription is thus achieved on application of the dimerizer. The system was originally developed by Ariad Pharmaceuticals to investigate gene function in eukaryotic cell systems, and the iDimerize system is now commercially available from Takara. To date, the iDimerize system has been

successfully used for basic research, including studies in *E. coli* (Roostaee *et al.*, 2009), *Saccharomyces cerevisiae* (Niu *et al.*, 2007), different cell cultures (Song *et al.*, 2007; Park *et al.*, 2012), *Caenorhabditis elegans* (Dossena *et al.*, 2011), *Mus musculus* (Shah *et al.*, 2007), *Xenopus laevis* (Isaacs *et al.*, 2007) and *Danio rerio* (Hirate and Okamoto, 2006), but has not been adapted for insects or pest control applications that involve transgenic insects.

2.3.3 GeneSwitch

The Gal4/UAS system is one of the most popular conditional gene expression systems available. It does not in itself result in inducible expression as delineated in this chapter, but an improvement has been developed. The GeneSwitch system is based on a chimeric Gal4 gene that encodes the GAL4 DNA-binding domain, the ligand-binding domain of the human progesterone receptor, and the activation domain from the human protein p65 (Osterwalder *et al.*, 2001). The chimeric molecule binds to the UAS sequence and activates transcription only in the presence of the antiprogestin RU486 (mifepristone). Hence, using tissue-specific promoters and activation by applying the ligand, the expression of UAS transgenes in both a temporally and a spatially specific manner is possible. This system has been demonstrated in *D. melanogaster*, facilitated by the availability of numerous extant fly lines carrying UAS sequences that are well characterized in terms of genetic environments and expression patterns (Poirier *et al.*, 2008). However, RU486 represses mitochondrial gene expression in *Drosophila*, a side effect that should be taken into consideration when designing experiments and interpreting the observed phenotypes (Robles-Murguia *et al.*, 2019).

GeneSwitch has been demonstrated in mammalian cells using a steroid or doxycycline (Ford *et al.*, 2007), or ethanol and mifepristone (Bhat *et al.*, 2004) to induce expression. The system has been combined with RNAi to achieve conditional expression

(Zhang *et al.*, 2010) and conditional inactivation (Ren *et al.*, 2009), overexpression for gene identification (Paik *et al.*, 2012) and tissue-specific alteration of gene expression (Robles-Murguia *et al.*, 2019) in *Drosophila*.

2.3.4 Q system

The Q transcriptional regulatory system, or QF-QUAS (Giles *et al.*, 1985, 1991; Potter *et al.*, 2010), of the filamentous fungus *Neurospora crassa* is involved in the metabolism of quinic acid as a carbon source in glucose-limiting conditions through a transcription factor (QA-1F, or QF) and the QUAS binding site located upstream of QF-regulated genes (Fig. 2.2). This interaction is repressed by the expression of QA-1S, or QS, or by quinic acid, in a feedback system whereby the molecule controls the expression of the genes responsible for its catabolism (Goll *et al.*, 2009; Subedi *et al.*, 2014).

In the absence of QF, low basal expression of QUAS-regulated genes is seen, though the level of QF-induced expression can be high (Potter *et al.*, 2010; Potter and Luo, 2011), allowing good control of transgene expression. Potter and Luo (2011) and colleagues (Potter *et al.*, 2010; Saibil, 2013) have characterized the use of the Q system in *Drosophila* experiments *in vivo* to express transgenic effectors in a tissue-specific manner and in combination with the GAL4 system for mosaic analysis, among other approaches. The Q system has also been used to drive temporally controlled, cell-specific expression in *C. elegans* (Potter *et al.*, 2010; Wei *et al.*, 2012). When separated, the QF and QUAS domains were transcribed only at a negligible level, but a significant level of expression was observed when combined.

The use of QF-QUAS as a binary repressible expression system was demonstrated in *Drosophila* and mammalian cells (Potter *et al.*, 2010; Subedi *et al.*, 2014). It was employed to determine cell division patterns and gene function and to investigate neurons involved in olfaction. *Drosophila* S2 cells transfected with QF and QUAS-luc2 showed 3300-fold greater expression than in the
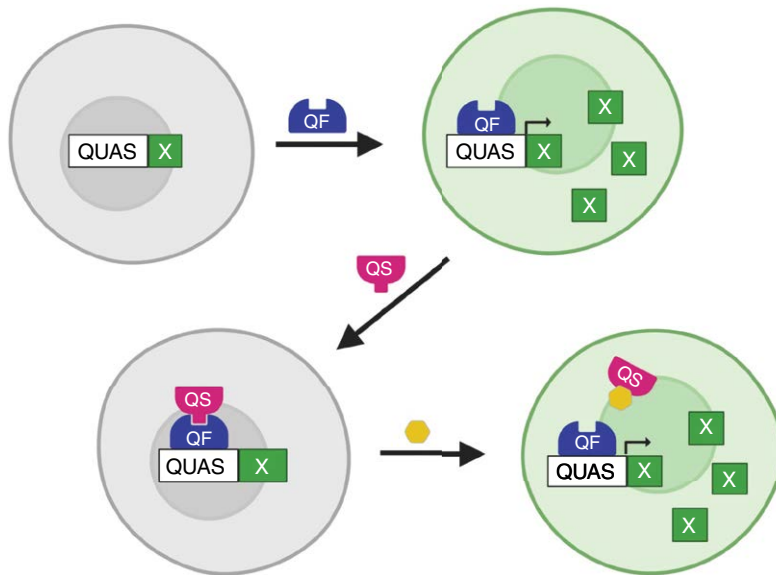
Fig. 2.2. The Q system. Three components comprise this system: the QF transcription factor, the QS suppressor, and QUAS-effectors. When QF binds to QUAS, target transgenes (X) are expressed. This can be silenced by ubiquitous expression of QS. Quinic acid can then be supplied to bind and inhibit the QS suppressor and can thus restore the transcription of the target transgene. Adapted from Potter *et al.*, 2010.

absence of QF, compared with a 5300-fold enhancement in the same system using GAL4 induction. Equal concentrations of QF and QS in the cells failed to achieve full suppression, which was QS-dose dependent, though QS suppression was achieved upon the addition of quinic acid. The crossing of separate transgenic lines of *Drosophila* carrying QUAS-driven markers, QF lines with specific promoters and ubiquitously driven QS lines demonstrated the functioning of the Q system *in vivo*: strong marker expression was observed in QF-QUAS flies, repressed by the introduction of QS (Potter and Luo, 2011; Wei *et al.*, 2012).

The system was shown to modulate gene expression in the first vertebrate organism: zebrafish, *Danio rerio* (Subedi *et al.*, 2014). A substantial reduction in expression of a transgene reporter under the control of tissue-specific promoters was achieved transiently by co-injection of the QS repressor into embryos. Tissue-specific activation of QUAS reporters by QF was shown by crossing transgenic QF driver lines to a QUAS:GFP reporter to be stable for three generations. The effect of quinic acid on gene expression was not tested.

The original QF consists of three structural domains: DBD (DNA binding and dimerization domain), MD (middle domain) and the transcriptional activation domain (AD). However, QF was found to be toxic in the *Drosophila* system (Riabinina *et al.*, 2015). Two variants of QF have been designed to avoid toxicity and maintain the functional activity of QF: the QF2 and QF2w. QF2 was designed by deleting the middle domain and was still fully capable of driving gene expression in *D. melanogaster* (Riabinina *et al.*, 2015). QF2w was further designed by changing the last two amino acids (glutamic acid and glutamine) of QF2 to four lysine(s) that change the charge on the C-terminus from negative to positive. This makes QF2w a weaker transcriptional activator but also less toxic. In addition, it can be more efficiently suppressed by QS than QF2. Temporal gene control can be achieved by controlling the amount of quinic acid fed to the flies, and the duration of exposure. *Drosophila* larvae are more receptive

to quinic acid in the food than adult flies. That makes it a better control agent for embryonic lethality control (Riabinina *et al.*, 2015). The Q system can also be combined with other expression systems to induce tightly controlled, specific and multi-gene expression. For example, it can be used together with Tet (Eckermann *et al.*, 2014; Mao *et al.*, 2019) and GAL4 systems (Potter *et al.*, 2010; Li and Stavropoulos, 2016). Alternatively, the expression of a transgene could be controlled by adding or removing quinic acid, providing an additional level of expression control and allowing the Q system to be incorporated into stable transgenic insects. Interestingly, although in *Drosophila* and *C. elegans* quinic acid blocks repression of QF by QS and thus allows expression of QUAS-regulated genes (Kawakami *et al.*, 2000; Wei *et al.*, 2012), in mammalian cells quinic acid enhances QF expression (Potter and Luo, 2011; Wei *et al.*, 2012). Quinic acid shows some toxicity in zebrafish: quinic acid added to rearing water at 0.5 mg/ml causes developmental abnormalities, though normal development can be achieved at 0.3 mg/ml (Subedi *et al.*, 2014), but appears to be non-toxic to insects. When cranberry juice, which contains over 1% quinic acid, was added to the food provided to *Drosophila* transformed with a QUAS-regulated transgene inhibited by QS expression, inhibition was reversed markedly within 24 h, allowing transgene expression (Potter *et al.*, 2010). No abnormalities were observed in flies reared for nine generations on cranberry juice media.

To date, the Q system has been successfully used in *D. melanogaster* (Potter and Luo, 2011), mammalian cells (Potter *et al.*, 2010), *C. elegans* (Maupas) (Wei *et al.*, 2012), *Danio rerio* (Hamilton) (Subedi *et al.*, 2014), *An. gambiae* (Riabinina *et al.*, 2016) and the plants *Glycine max* and *Nicotiana benthamiana* (Persad *et al.*, 2020). The complexity and amount of regulating elements of the Q system can be an advantage for different application scenarios. On the other hand, those factors have to be transformed, evaluated and optimized to achieve the tight control of expression that is required for any pest control application.

2.3.5 Use of Cre/*loxP* recombination

Another system that allows the inducible or suppressible transgene expression in a reversible manner is the Cre/*loxP* site-specific DNA recombination system (see Ahmed and Wimmer, Chapter 5, this volume). The *Cre* gene of the P1 bacteriophage encodes a site-specific recombinase that recombines a pair of short target sequences called the *lox* sequences. This technology can be used to introduce a particular gene into the genome to study its function. Alternatively, it is possible to use an inducible *Cre* with specific promoters to produce transgenic expression at a specific time and in a specific tissue. Various laboratories have integrated the tetracycline system with a Cre/*loxP* to achieve inducible expression (Sun *et al.*, 2007; Bertram *et al.*, 2009) or conditional DNA recombination (Hennighausen *et al.*, 1995; Guo *et al.*, 2005). Gene expression has been modulated in various species using FLP- and Cre-recombinase-mediated excision or recombination as well as the phiC31 integrase system (see Ahmed and Wimmer, Chapter 5, this volume for detailed information).

2.4 Conclusions

While a variety of inducible systems have been described, there is still a lack of suitable systems for insects that provide a range of levels of controllable induction or repression and negligible constitutive expression in the non-induced or repressed states. The phenotype required constrains the possible choices from among those discussed above: a low level of expression might be acceptable for some effectors, but stringent control is essential when toxicity or mutagenic effects are expected. As methods for systematically analysing transcriptomes and genomes develop further, valuable new promoters will be identified as non-model insects are exposed to potentially inducing and repressing treatments. The examples we have described provide several improvements to transgenes that have been tested in insects as well as candidates for further exploration outside of those model organisms in which they have been developed.

References

Amin, J., Mestril, R., Schiller, P., Dreano, M. and Voellmy, R. (1987) Organization of the *Drosophila melanogaster hsp70* heat shock regulation unit. *Molecular and Cellular Biology* 7, 1055–1062.

Andersen, R.D., Taplitz, S.J., Wong, S., Bristol, G., Larkin, B. and Herschman, H.R. (1987) Metal-dependent binding of a factor in vivo to the metal-responsive elements of the *metallothionein 1* gene promoter. *Molecular and Cellular Biology* 7, 3574–3581.

Aumann, R.A., Hacker, I. and Schetelig, M.F. (2020) Female-to-male sex conversion in *Ceratitis capitata* by CRISPR/Cas9 HDR-induced point mutations in the sex determination gene *transformer-2*. *Scientific Reports* 10, 18611.

Belote, J.M. and Baker, B.S. (1982) Sex determination in *Drosophila melanogaster*: analysis of *transformer-2*, a sex-transforming locus. *Proceedings of the National Academy of Sciences USA* 79, 1568–1572.

Benedict, M.Q., Cockburn, A.F. and Seawright, J.A. (1991) Heat-shock mortality and induced thermotolerance in larvae of the mosquito *Anopheles albimanus*. *Journal of the American Mosquito Control Association* 7, 547–550.

Benkel, B.F. and Hickey, D.A. (1986) Glucose repression of amylase gene expression in *Drosophila melanogaster*. *Genetics* 114, 137–144.

Benkel, B.F. and Hickey, D.A. (1987) A *Drosophila* gene is subject to glucose repression. *Proceedings of the National Academy of Sciences USA* 84, 1337–1339.

Bertram, R., Kolb, M. and Hillen, W. (2009) In vivo activation of tetracycline repressor by Cre/lox-mediated gene assembly. *Journal of Molecular and Microbiolial Biotechnology* 17, 136–145.

Bhat, R.A., Stauffer, B., Komm, B.S. and Bodine, P.V. (2004) Regulated expression of sFRP-1 protein by the GeneSwitch system. *Protein Expression and Purification* 37, 327–335.

Bienz, M. and Pelham, H.R. (1987) Mechanisms of heat-shock gene activation in higher eukaryotes. *Advances in Genetics* 24, 31–72.

Blevins, J.S., Revel, A.T., Smith, A.H., Bachlani, G.N. and Norgard, M.V (2007) Adaptation of a luciferase gene reporter and lac expression system to *Borrelia burgdorferi*. *Applied and Environmental Microbiology* 73, 1501–1513.

Boneca, I.G., Ecobichon, C., Chaput, C., Mathieu, A., Guadagnini, S. *et al.* (2008) Development of inducible systems to engineer conditional mutants of essential genes of *Helicobacter pylori*. *Applied and Environmental Microbiology* 74, 2095–2102.

Bonner, J.J., Parks, C., Parker-Thornburg, J., Mortin, M.A. and Pelham, H.R. (1984) The use of promoter fusions in *Drosophila* genetics: isolation of mutations affecting the heat shock response. *Cell* 37, 979–991.

Burnett, S.H., Kershen, E.J., Zhang, J., Zeng, L., Straley, S.C. *et al.* (2004) Conditional macrophage ablation in transgenic mice expressing a Fas-based suicide gene. *Journal of Leukocyte Biology* 75, 612–623.

Caron, L., Prot, M., Rouleau, M., Rolando, M., Bost, F. and Binétruy, B. (2005) The Lac repressor provides a reversible gene expression system in undifferentiated and differentiated embryonic stem cell. *Cellular and Molecular Life Sciences* 62, 1605–1612.

Carpenetti, T.L., Aryan, A., Myles, K.M. and Adelman, Z.N.(2012) Robust heat-inducible gene expression by two endogenous *hsp70*-derived promoters in transgenic *Aedes aegypti*. *Insect Molecular Biology* 21, 97–106.

Carvalho, D.O., McKemey, A.R., Garziera, L., Lacroix, R., Donnelly, C.A. *et al.* (2015) Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. *PLoS Neglected Tropical Diseases* 9, e0003864.

Chen, B., Hrycaj, S., Schinko, J.B., Podlaha, O., Wimmer, E.A. *et al.* (2011) Pogostick: a new versatile *piggyBac* vector for inducible gene over-expression and down-regulation in emerging model systems. *PLoS ONE* 6, e18659.

Chen, M.S., Obar, R.A., Schroeder, C.C., Austin, T.W., Poodry, C.A. *et al.* (1991) Multiple forms of dynamin are encoded by *shibire*, a *Drosophila* gene involved in endocytosis. *Nature* 351, 583–586.

Choo, A., Fung, E., Chen, I.Y., Saint, R., Crisp, P. and Baxter, S.W. (2020) Precise single base substitution in the *shibire* gene by CRISPR/Cas9-mediated homology directed repair in *Bactrocera tryoni*. *BMC Genetics* 21, 127.

Concha, C., Palavesam, A., Guerrero, F.D., Sagel, A., Li, F. *et al.* (2016) A transgenic male-only strain of the New World screwworm for an improved control program using the sterile insect technique. *BMC Biology* 14, 72.

Coyle, P., Philcox, J.C., Carey, L.C. and Rofe, A.M. (2002) Metallothionein: the multipurpose protein. *Cellular and Molecular Life Sciences* 59, 627–647.

Cronin, C.A., Gluba, W. and Scrable, H. (2001) The lac operator-repressor system is functional in the mouse. *Genes & Development* 15, 1506–1517.

Dai, H., Jiang, R., Wang, J., Xu, G., Cao, M., Wang, Z. and Fei, J. (2007) Development of a heat shock inducible and inheritable RNAi system in silkworm. *Biomolecular Engineering* 24, 625–630.

de Boer, H.A., Comstock, L.J. and Vasser, M. (1983) The *tac* promoter: a functional hybrid derived from the *trp* and *lac* promoters. *Proceedings of the National Academy of Sciences USA* 80, 21–25.

de Lorenzo, V., Eltis, L., Kessler, B. and Timmis, K.N. (1993) Analysis of *Pseudomonas* gene products using lacIq/Ptrp-lac plasmids and transposons that confer conditional phenotypes. *Gene* 123, 17–24.

Dossena, S., Gandini, R., Tamma, G., Vezzoli, V., Nofziger, C. *et al.* (2011) The molecular and functional interaction between ICln and HSPC038 proteins modulates the regulation of cell volume. *Journal of Biological Chemistry* 286, 40659–40670.

Eckermann, K.N., Dippel, S., KaramiNejadRanjbar, M., Ahmed, H.M., Curril, I.M. and Wimmer, E.A. (2014) Perspective on the combined use of an independent transgenic sexing and a multifactorial reproductive sterility system to avoid resistance development against transgenic Sterile Insect Technique approaches. *BMC Genetics* 15 (Suppl. 2), S17

Ford, D., Hoe, N., Landis, G.N., Tozer, K., Luu, A., Bhole, D., Badrinath, A. and Tower, J. (2007) Alteration of *Drosophila* life span using conditional, tissue-specific expression of transgenes triggered by doxycyline or RU486/Mifepristone. *Experimental Gerontology* 42, 483–497.

Franz, G. (2005) Genetic sexing strains in Mediterranean fruit fly, an example for other species amenable to large-scale rearing for the sterile insect technique. In: Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) *Sterile Insect Technique: Principles and Practice in Area-wide Integrated Pest Management*. Springer, Dordrecht, Netherlands, pp. 427–451.

Franz, G., Bourtzis, K. and Caceres-Barrios, C. (2021) Practical and operational genetic sexing systems based on classical genetic approaches in fruit flies, an example for other species amenable to large-scale rearing for the sterile insect technique. In: Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management*, 2nd edn. CRC Press, Boca Raton, Florida, pp. 575–604.

Fu, G., Lees, R.S., Nimmo, D., Aw, D., Jin, L., Gray, P., Berendonk, T.U., White-Cooper, H., Scaife, S., Kim Phuc, H., Marinotti, O., Jasinskiene, N., James, A.A. and Alphey, L. (2010) Female-specific flightless phenotype for mosquito control. *Proceedings of the National Academy of Sciences USA* 107, 4550–4554.

Gamez, S., Vesga, L.C., Mendez-Sanchez, S.C. and Akbari, O.S. (2021) Spatial control of gene expression in flies using bacterially derived binary transactivation systems. *Insect Molecular Biology* 30(5), 461–471. doi: 10.1111/imb.12717

Giles, N.H., Case, M.E., Baum, J., Geever, R., Huiet, L. *et al.* (1985) Gene organization and regulation in the qa (quinic acid) gene cluster of *Neurospora crassa*. *Microbiological Reviews* 49, 338–358.

Giles, N.H., Geever, R.F., Asch, D.K., Avalos, J. and Case, M.E. (1991) Organization and regulation of the qa (quinic acid) genes in *Neurospora crassa* and other fungi. *The Journal of Heredity* 82, 1–7.

Goll, M.G., Anderson, R., Stainier, D.Y., Spradling, A.C. and Halpern, M.E. (2009) Transcriptional silencing and reactivation in transgenic zebrafish. *Genetics* 182, 747–755.

Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences USA* 89(12), 5547–5551.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W. and Bujard, H. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science* 268, 1766–1769.

Grespi, F., Ottina, E., Yannoutsos, N., Geley, S. and Villunger, A. (2011) Generation and evaluation of an IPTG-regulated version of *Vav*-gene promoter for mouse transgenesis. *PloS ONE* 6, e18051.

Grigliatti, T.A., Hall, L., Rosenbluth, R. and Suzuki, D.T. (1973) Temperature-sensitive mutations in *Drosophila melanogaster*. XIV. A selection of immobile adults. *Molecular Genomics and Genetics* 120, 107–114.

Gross, T.L., Myles, K.M. and Adelman, Z.N. (2009) Identification and characterization of heat shock 70 genes in *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology* 46, 496–504.

Guo, Z.M., Xu, K., Yue, Y., Huang, B., Deng, X.Y. *et al.* (2005) Temporal control of Cre recombinase-mediated in vitro DNA recombination by Tet-on gene expression system. *Acta Biochimica et Biophysica Sinica* 37, 133–138.

Harris, A.F., McKemey, A.R., Nimmo, D., Curtis, Z., Black, I. *et al.* (2012) Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nature Biotechnology* 30, 828–830.

Heinrich, J.C. and Scott, M.J. (2000) A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. *Proceedings of the National Academy of Sciences USA* 97, 8229–8232.

Hennighausen, L., Wall, R.J., Tillmann, U., Li, M. and Furth, P.A. (1995) Conditional gene expression in secretory tissues and skin of transgenic mice using the MMTV-LTR and the tetracycline responsive system. *Journal of Cellular Biochemistry* 59, 463–472.

Hess, M.A. and Duncan, R.F. (1996) Sequence and structure determinants of *Drosophila Hsp*70 mRNA translation: 5'UTR secondary structure specifically inhibits heat shock protein mRNA translation. *Nucleic Acids Research* 24, 2441–2449.

Hickey, D.A., Benkel, K.I., Fong, Y. and Benkel, B.F. (1994) A *Drosophila* gene promoter is subject to glucose repression in yeast cells. *Proceedings of the National Academy of Sciences USA* 91, 11109–11112.

Hirate, Y. and Okamoto, H. (2006) Canopy1, a novel regulator of FGF signaling around the midbrain-hindbrain boundary in zebrafish. *Current Biology* 16, 421–427.

Horn, C. and Wimmer, E.A. (2003) A transgene-based, embryo-specific lethality system for insect pest management. *Nature Biotechnology* 21, 64–70.

Isaacs, H.V., Deconinck, A.E. and Pownall, M.E. (2007) FGF4 regulates blood and muscle specification in *Xenopus laevis*. *Biology of the Cell* 99, 165–173.

Jaffri, S., Yan, Y., Scott, M. and Schetelig, M. (2020) Conditional expression systems for *Drosophila suzukii* pest control. In: Garcia, F. (ed.) *Drosophila suzukii Management.* Springer, Cham, Switzerland, pp. 195–215.

Kalosaka, K., Chrysanthis, G., Rojas-Gill, A.P., Theodoraki, M., Gourzi, P. *et al.* (2006) Evaluation of the activities of the medfly and *Drosophila hsp*70 promoters in vivo in germ-line transformed medflies. *Insect Molecular Biology* 15, 373–382.

Kalosaka, K., Soumaka, E., Politis, N. and Mintzas, A.C. (2009) Thermotolerance and *hsp*70 expression in the Mediterranean fruit fly *Ceratitis capitata*. *Journal of Insect Physiology* 55, 568–573.

Kawakami, K., Shima, A. and Kawakami, N. (2000) Identification of a functional transposase of the *Tol2* element, an *Ac*-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage. *Proceedings of the National Academy of Sciences USA* 97, 11403–11408.

Khlebnikov, A. and Keasling, J.D. (2002) Effect of *lacY* expression on homogeneity of induction from the P_{tac} and P_{trc} promoters by natural and synthetic inducers. *Biotechnology Progress* 18, 672–674.

Klemenz, R., Hultmark, D. and Gehring, W.J. (1985) Selective translation of heat shock mRNA in *Drosophila melanogaster* depends on sequence information in the leader. *The EMBO Journal* 4, 2053–2060.

Klemm, J.D., Schreiber, S.L. and Crabtree, G.R. (1998) Dimerization as a regulatory mechanism in signal transduction. *Annual Review of Immunology* 16, 569–592.

Kovach, M.J., Carlson, J.O. and Beaty, B.J. (1992) A *Drosophila* metallothionein promoter is inducible in mosquito cells. *Insect Molecular Biology* 1, 37–43.

Lange, C., Hemmrich, G., Klostermeier, U.C., Lopez-Quintero, J.A., Miller *et al.* (2011) Defining the origins of the NOD-like receptor system at the base of animal evolution. *Molecular Biology and Evolution* 28, 1687–1702.

Letouvet-Pawlak, B., Monnier, C., Barray, S., Hodgson, D.A. and Guespin-Michel, J.F. (1990) Comparison of beta-galactosidase production by two inducible promoters in *Myxococcus xanthus*. *Research in Microbiology* 141, 425–435.

Lewandoski, M. (2001) Conditional control of gene expression in the mouse. *Nature Reviews. Genetics* 2, 743–755.

Li, J. and Handler, A.M. (2017) Temperature-dependent sex-reversal by a *transformer-2* gene-edited mutation in the spotted wing drosophila, *Drosophila suzukii. Scientific Reports* 7, 12363.

Li, Q. and Stavropoulos, N. (2016) Evaluation of ligand-inducible expression systems for conditional neuronal manipulations of sleep in *Drosophila*. *G3: Genes, Genomes, Genetics* 6, 3351–3359.

Lin, H.H., Yang, T.P., Jiang, S.T., Liu, H.S. and Tang, M.J. (1997) Inducible expression of bcl-2 by the lac operator/repressor system in MDCK cells. *American Journal of Physiology-Renal Physiology* 273, F300–306.

Lindquist, S. (1986) The heat-shock response. *Annual Review of Biochemistry* 55, 1151–1191.

Lindquist, S. and Petersen, R. (1990) Selective translation and degradation of heat-shock messenger RNAs in *Drosophila*. *Enzyme* 44, 147–166.

Lis, J.T., Simon, J.A. and Sutton, C.A. (1983) New heat shock puffs and beta-galactosidase activity resulting from transformation of *Drosophila* with an *hsp70-lacZ* hybrid gene. *Cell* 35, 403–410.

Liu, H.S., Lee, C.H., Lee, C.F., Su, I.J. and Chang, T.Y. (1998) Lac/Tet dual-inducible system functions in mammalian cell lines. *Biotechniques* 24, 624–628, 630–622.

Lycett, G.J., Kafatos, F.C. and Loukeris, T.G. (2004) Conditional expression in the malaria mosquito *Anopheles stephensi* with Tet-On and Tet-Off systems. *Genetics* 167, 1781–1790.

Makrides, S.C. (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiological Reviews* 60, 512–538.

Mao, S., Qi, Y., Zhu, H., Huang, X., Zou, Y. and Chi, T. (2019) A Tet/Q hybrid system for robust and versatile control of transgene expression in *C. elegans.* iScience 11, 224–237.

Maroni, G., Wise, J., Young, J.E. and Otto, E. (1987) Metallothionein gene duplications and metal tolerance in natural populations of *Drosophila melanogaster*. *Genetics* 117, 739–744.

Miller, L.H., Sakai, R.K., Romans, P., Gwadz, R.W., Kantoff, P. and Coon, H.G. (1987) Stable integration and expression of a bacterial gene in the mosquito *Anopheles gambiae. Science* 237, 779–781.

Morimoto, R.I. (1993) Cells in stress: transcriptional activation of heat shock genes. *Science* 259, 1409–1410.

Morrison, N.I., Simmons, G.S., Fu, G., O'Connell, S., Walker, A.S. *et al.* (2012) Engineered repressible lethality for controlling the pink bollworm, a lepidopteran pest of cotton. *PloS ONE* 7, e50922.

Nirmala, X., Zimowska G.J. and Handler, A.M. (2009) Characterization of the proteasome beta2 subunit gene and its mutant allele in the tephritid fruit fly pest, *Anastrepha suspensa*. *Insect Molecular Biology* 18, 330–334.

Niu, H., Li, X., Job, E., Park, C., Moazed, D. *et al.* (2007) Mek1 kinase is regulated to suppress double-strand break repair between sister chromatids during budding yeast meiosis. *Molecular and Cellular Biology* 27, 5456–5467.

O'Donnell, J., Gerace, L., Leister, F. and Sofer, W. (1975) Chemical selection of mutants that affect alcohol dehydrogenase in *Drosophila*. II. Use of 1-pentyne-3-ol. *Genetics* 79, 73–83.

Oberhofer, G., Ivy, T. and Hay, B.A. (2021) Split versions of Cleave and Rescue selfish genetic elements for measured self limiting gene drive. *PLoS Genetics* 17, e1009385.

Ogaugwu, C.E., Schetelig, M.F. and Wimmer, E.A. (2013) Transgenic sexing system for *Ceratitis capitata* (Diptera: Tephritidae) based on female-specific embryonic lethality. *Insect Biochemistry and Molecular Biology* 43, 1–8.

Osterwalder, T., Yoon, K.S., White, B.H. and Keshishian, H. (2001) A conditional tissue-specific transgene expression system using inducible GAL4. *Proceedings of the National Academy of Sciences USA* 98, 12596–12601.

Otto, E., Allen, J.M., Young, J.E., Palmiter, R.D. and Maroni, G. (1987) A DNA segment controlling metal-regulated expression of the *Drosophila melanogaster* metallothionein gene Mtn. *Molecular and Cellular Biology* 7, 1710–1715.

Paik, D., Jang, Y.G., Lee, Y.E., Lee, Y.N., Yamamoto, R. *et al.* (2012) Misexpression screen delineates novel genes controlling *Drosophila* lifespan. *Mechanisms of Ageing and Development* 133, 234–245.

Papadimitriou, E., Kritikou, D., Mavroidis, M., Zacharopoulou, A. and Mintzas, A.C. (1998) The heat shock 70 gene family in the Mediterranean fruit fly *Ceratitis capitata*. *Insect Molecular Biology* 7, 279–290.

Park, J., Sarode, V.R., Euhus, D., Kittler, R. and Scherer, P.E. (2012) Neuregulin 1-HER axis as a key mediator of hyperglycemic memory effects in breast cancer. *Proceedings of the National Academy of Sciences USA* 109, 21058–21063.

Parsell, D.A. and Lindquist, S. (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annual Review of Genetics* 27, 437–496.

Patil, N.S., Lole, K.S. and Deobagkar, D.N. (1996) Adaptive larval thermotolerance and induced cross-tolerance to propoxur insecticide in mosquitoes *Anopheles stephensi* and *Aedes aegypti*. *Medical and Veterinary Entomology* 10, 277–282.

Pelham, H.R. (1982) A regulatory upstream promoter element in the *Drosophila hsp70* heat-shock gene. *Cell* 30, 517–528.

Persad, R., Reuter, D.N., Dice, L.T., Nguyen, M.A., Rigoulot, S.B. *et al.* (2020) The Q-System as a synthetic transcriptional regulator in plants. *Frontiers in Plant Science* 11, 245.

Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J. *et al.* (2007) Late-acting dominant lethal genetic systems and mosquito control. *BMC Biology* 5, 11.

Poirier, L., Shane, A., Zheng, J. and Seroude, L. (2008) Characterization of the *Drosophila* gene-switch system in aging studies: a cautionary tale. *Aging Cell* 7, 758–770.

Pollock, R. and Clackson, T. (2002) Dimerizer-regulated gene expression. *Current Opinion in Biotechnology* 13, 459–467.

Potter, C.J. and Luo, L. (2011) Using the Q system in *Drosophila melanogaster*. *Nature Protocols* 6, 1105–1120.

Potter, C.J., Tasic, B., Russler, E.V., Liang, L. and Luo, L. (2010) The Q system: a repressible binary system for transgene expression, lineage tracing, and mosaic analysis. *Cell* 141, 536–548.

Ren, C., Finkel, S.E. and Tower, J. (2009) Conditional inhibition of autophagy genes in adult *Drosophila* impairs immunity without compromising longevity. *Experimental Gerontology* 44, 228–235.

Riabinina, O., Luginbuhl, D., Marr, E., Liu, S., Wu, M.N. *et al.* (2015) Improved and expanded Q-system reagents for genetic manipulations. *Nature Methods* 12, 219–225.

Riabinina, O., Task, D., Marr, E., Lin, C.C., Alford, R. *et al.* (2016) Organization of olfactory centres in the malaria mosquito *Anopheles gambiae*. *Nature Communications* 7, 13010.

Robinson, A.S. (2002) Genetic sexing strains in medfly, *Ceratitis capitata*, sterile insect technique programmes. *Genetica* 116, 5–13.

Robles-Murguia, M., Hunt, L.C., Finkelstein, D., Fan, Y. and Demontis, F. (2019) Tissue-specific alteration of gene expression and function by RU486 and the GeneSwitch system. *NPJ and Mechanisms of Disease* 5, 6.

Roostaee, A., Cote, S. and Roucou, X. (2009) Aggregation and amyloid fibril formation induced by chemical dimerization of recombinant prion protein in physiological-like conditions. *The Journal of Biological Chemistry* 284, 30907–30916.

Saibil, H. (2013) Chaperone machines for protein folding, unfolding and disaggregation. *Nature Reviews Molecular Cell Biology* 14, 630–642.

Sakai, R.K. and Miller, L.H. (1992) Effects of heat shock on the survival of transgenic *Anopheles gambiae* (Diptera: Culicidae) under antibiotic selection. *Journal of Medical Entomology* 29, 374–375.

Sakurai, H. and Enoki, Y. (2010) Novel aspects of heat shock factors: DNA recognition, chromatin modulation and gene expression. *The FEBS Journal* 277, 4140–4149.

Schetelig, M.F. and Handler, A.M. (2012a) Strategy for enhanced transgenic strain development for embryonic conditional lethality in *Anastrepha suspensa*. *Proceedings of the National Academy of Sciences USA* 109, 9348–9353.

Schetelig, M.F. and Handler, A.M. (2012b) A transgenic embryonic sexing system for *Anastrepha suspensa* (Diptera: Tephritidae). *Insect Biochemistry and Molecular Biology* 42, 790–795

Schetelig, M.F., Caceres, C., Zacharopoulou, A., Franz, G. and Wimmer, E.A. (2009) Conditional embryonic lethality to improve the sterile insect technique in *Ceratitis capitata* (Diptera: Tephritidae). *BMC Biology* 7, 4.

Schetelig, M.F., Targovska, A., Meza, J.S., Bourtzis, K. and Handler, A.M. (2016) Tetracycline-suppressible female lethality and sterility in the Mexican fruit fly, *Anastrepha ludens*. *Insect Molecular Biology* 25, 500–508.

Schetelig, M.F., Schwirz, J. and Yan, Y. (2021) A transgenic female killing system for the genetic control of *Drosophila suzukii*. *Scientific Reports* 11, 12938.

Schweisguth, F., Lepesant, J.A. and Vincent, A. (1990) The serendipity alpha gene encodes a membrane-associated protein required for the cellularization of the *Drosophila* embryo. *Genes & Development* 4, 922–931.

Shah, V.R., Koster, M.I., Roop, D.R., Spencer, D.M., Wei, L. *et al.* (2007) Double-inducible gene activation system for caspase 3 and 9 in epidermis. *Genesis* 45, 194–199.

Smyth, K.A. and Belote, J.M. (1999) The dominant temperature-sensitive lethal DTS7 of *Drosophila melanogaster* encodes an altered 20S proteasome beta-type subunit. *Genetics* 151, 211–220.

Song, G.J., Jones, B.W. and Hinkle, P.M. (2007) Dimerization of the thyrotropin-releasing hormone receptor potentiates hormone-dependent receptor phosphorylation. *Proceedings of the National Academy of Sciences USA* 104, 18303–18308.

Spencer, D.M., Wandless, T.J., Schreiber, S.L. and Crabtree, G.R. (1993) Controlling signal transduction with synthetic ligands. *Science* 262, 1019–1024.

Steller, H. and Pirrotta, V. (1985a) Expression of the *Drosophila* white gene under the control of the *hsp70* heat shock promoter. *The EMBO Journal* 4, 3765–3772.

Steller, H. and Pirrotta, V. (1985b) A transposable P vector that confers selectable G418 resistance to *Drosophila* larvae. *The EMBO Journal* 4, 167–171.

Storti, R.V., Scott, M.P., Rich, A. and Pardue, M.L. (1980) Translational control of protein synthesis in response to heat shock in *Drosophila melanogaster* cells. *Cell* 22, 825–834.

Subedi, A., Macurak, M., Gee, S.T., Monge, E., Goll, M.G. *et al.* (2014) Adoption of the Q transcriptional regulatory system for zebrafish transgenesis. *Methods* 66, 433–440.

Sun, Y., Chen, X. and Xiao, D. (2007) Tetracycline-inducible expression systems: new strategies and prac-
tices in the transgenic mouse modeling. *Acta Biochimica et Biophysica Sinica* 39, 235–246.

Tachibana, S., Numata, H. and Goto, S.G. (2005) Gene expression of heat-shock proteins (*Hsp23*, *Hsp70*
and *Hsp90*) during and after larval diapause in the blow fly *Lucilia sericata*. *Journal of Insect Physi-
ology* 51, 641–647.

Thomas, D.D., Donnelly, C.A., Wood, R.J. and Alphey, L.S. (2000) Insect population control using a domin-
ant, repressible, lethal genetic system. *Science* 287, 2474–2476.

Tissieres, A., Mitchell, H.K. and Tracy, U.M. (1974) Protein synthesis in salivary glands of *Drosophila mela-
nogaster*: relation to chromosome puffs. *Journal of Molecular Biology* 84, 389–398.

Uhlirova, M., Asahina, M., Riddiford, L.M. and Jindra, M. (2002) Heat-inducible transgenic expression in the
silkmoth *Bombyx mori*. *Development Genes and Evolution* 212, 145–151.

van der Bliek, A.M. and Meyerowitz, E.M. (1991) Dynamin-like protein encoded by the *Drosophila shibire*
gene associated with vesicular traffic. *Nature* 351, 411–414.

Vivinus, S., Baulande, S., van Zanten, M., Campbell, F., Topley, P. *et al.* (2001) An element within the 5' un-
translated region of human *Hsp70* mRNA which acts as a general enhancer of mRNA translation.
European Journal of Biochemistry 268, 1908–1917.

Voellmy, R. and Rungger, D. (1982) Transcription of a *Drosophila* heat shock gene is heat-induced in *Xen-
opus oocytes*. *Proceedings of the National Academy of Sciences USA* 79, 1776–1780.

Wakiyama, M., Muramatsu, R., Kaitsu, Y., Ikeda, M and Yokoyama, S. (2011) Inducible protein expression
in *Drosophila* Schneider 2 cells using the *lac* operator-repressor system. *Biotechnology Letters* 33,
2361–2366.

Wei, X., Potter, C.J., Luo, L. and Shen, K. (2012) Controlling gene expression with the Q repressible binary
expression system in *Caenorhabditis elegans*. *Nature Methods* 9, 391–395.

Whited, J.L., Lehoczky, J.A. and Tabin, C.J. (2012) Inducible genetic system for the axolotl. *Proceedings of
the National Academy of Sciences USA* 109, 13662–13667.

Wing, D., Koncz, C. and Schell, J. (1989) Conserved function in *Nicotiana tabacum* of a single *Drosophila
hsp*70 promoter heat shock element when fused to a minimal T-DNA promoter. *Molecular and Gen-
eral Genetics* 219, 9–16.

Wu, J.D., Hsueh, H.C., Huang, W.T., Liu, H.S., Leung, H.W. *et al.* (1997) The inducible lactose operator-
repressor system is functional in the whole animal. *DNA and Cell Biology* 16, 17–22.

Xue, G.P., Johnson, J.S., Smyth, D.J., Dierens, L.M., Wang, X. *et al.* (1996) Temperature-regulated ex-
pression of the tac/lacl system for overproduction of a fungal xylanase in *Escherichia coli*. *Applied
Microbiology and Biotechnology* 45, 120–126.

Yan, Y., Kobayashi, Y., Huang, C., Liu, B., Qian, W. *et al.* (2021) Highly efficient temperature inducible CRIS-
PR-Cas9 gene targeting in *Drosophila suzukii*. *International Journal of Molecular Sciences* 22, 6724.

Yan, Y. and Scott, M.J. (2015) A transgenic embryonic sexing system for the Australian sheep blow fly *Lucil-
ia cuprina*. *Scientific Reports* 5, 16090.

Zeidler, M.P., Tan, C., Bellaiche, Y., Cherry, S., Hader, S. *et al.* (2004) Temperature-sensitive control of
protein activity by conditionally splicing inteins. *Nature Biotechnology* 22, 871–876.

Zhang, H., Li, Y., Yang, J., Tominaga, K., Pereira-Smith, O.M. and Tower, J. (2010) Conditional inactivation
of MRG15 gene function limits survival during larval and adult stages of *Drosophila melanogaster*.
Experimental Gerontology 45, 825–833.

Zhao, L. and Jones, W. (2012) Expression of heat shock protein genes in insect stress respones. *Inverte-
brate Survival Journal* 9, 93–101.

Zhu, Z. and Thiele, D.J. (1996) Toxic metal-responsive gene transcription. *Experientia Supplementum (EXS)*
77, 307–320.

Zhu, Z., Zheng, T., Lee, C.G., Homer, R.J. and Elias, J.A. (2002) Tetracycline-controlled transcriptional
regulation systems: advances and application in transgenic animal modeling. *Seminars in Cell &
Developmental Biology* 13, 121–128.

3 Sex-, Tissue- and Stage-Specific Transgene Expression

Tony Nolan[1]* and Andrew M. Hammond[2]

[1]*Liverpool School of Tropical Medicine: Department of Vector Biology;* [2]*Johns Hopkins Bloomberg School of Public Health | JHSPH: W. Harry Feinstone Department of Molecular Microbiology and Immunology*

3.1 Introduction

Transgenesis is perhaps the most powerful tool in the hands of researchers hoping to dissect the molecular pathways underlying the biology of insects and their interactions with pathogens and the microbiome. The past decade has also seen a dramatic shift towards applied transgenesis to exert genetic control of pest and vector species by incorporating effector transgenes designed to modify or suppress target populations (Wang *et al.*, 2021). Critical to these efforts is the controlled activity of transgenes over space and time – ranging from cell and tissue specificity to broadly active drivers in a particular stage or sex. This chapter aims to describe the methods and technologies used to exert spatio-temporal control of transgene expression in insects, with an emphasis on mosquitoes, and to illustrate how these have been implemented for research and genetic control.

3.2 Gene Regulation in Insects

Regulation of gene expression can occur at any level as information flows from DNA to mRNA and protein, including post-translational modifications that can affect the precise level and specificity of expression within cells and tissues. Controlling this expression is complex in insects, as with other eukaryotes, and relies upon the interaction of *cis*-acting factors (i.e., regulatory DNA sequences and epigenetic modifications in close proximity to the gene) and *trans*-acting factors (i.e., distally encoded DNA, RNA and protein that interact with *cis*-acting sequences), many of which are sex or cell-type specific. These are in turn affected by cues that may be endogenous, such as circadian, developmental, behavioural and hormone signalling, or from externally derived stimuli such as mating, feeding, gut microbiota, environmental conditions and exposure to chemicals.

3.2.1 Transcriptional control

Perhaps the most important and best-understood mechanism of gene regulation is at the level of transcription, controlled primarily by the interaction between RNA polymerase and transcription factors (TFs) at non-coding *cis*-acting DNA elements: promoters, enhancers, silencers and chromatin modulators. Transcription factors are

---

*Corresponding author, email: Tony.Nolan@lstmed.ac.uk

themselves present in specific subsets of cells or tissues and it is the combined binding of these factors to their cognate transcription factor binding site (TFBS) that drives unique and precise profiles of transcription. The number, nature and strength of these interactions determines the specificity and intensity of transcription whereby promoters and enhancers serve to recruit RNA polymerase, but silencers limit expression by recruiting suppressor promoters (reviewed in Zabidi and Stark, 2016).

3.2.2   The promoter

The promoter is a segment of DNA located immediately upstream of a gene that contains the primary signals to initiate gene expression and is often sufficient to drive transgene expression with spatio-temporal specificity. Promoters consist of three distinct regions: the core promoter, proximal promoter and distal promoter. Transcription is initiated at the core promoter, a sequence typically several hundred bases in length comprising the transcription start site (TSS), a binding site for RNA polymerase, and core TFBSs such as the TATA box and B-recognition element – which together promote formation of the pre-initiation complex. Immediately upstream of this sits the proximal promoter, which is decorated with specific TFBSs to exert the most important influence over spatio-temporal dynamics of transcription. The distal promoter is located further upstream and contains typically weak TFBSs that exert minimal influence over gene expression as compared with the proximal promoter (reviewed in Danino *et al*., 2015; Zabidi and Stark, 2016). Many endogenous promoters have been isolated, characterized and used to drive transgene expression in insects and examples of these are collated in Table 3.1.

3.2.3   Enhancers and silencers

Enhancers are *cis*-regulatory elements that act similarly to the distal promoter, but can function independent of orientation, distance, or location with respect to the target gene. Typically located in intergenic regions, they are decorated by TFBSs, and physically locate cognate transcription factors to the promoter region – either by proximity with the promoter, or by forming loops during the activation of transcription. Further interactions with cell type-specific TFs, chromatin modifiers, co-regulators, architectural proteins and RNA polymerase lead to modified gene expression that can show extreme cell-type specificity (reviewed in Shaul, 2017; Panigrahi and O'Malley, 2021). Silencers can be thought of as the lesser-known repressive counterparts of enhancers, mediating gene expression by recruiting repressive elements in much the same manner (reviewed in Segert *et al*., 2021). Whilst efforts to identify insect enhancer elements are ongoing (Nardini *et al*., 2019; Schember and Halfon, 2021) they are not usually incorporated into transgenic expression constructs by design, because few have been defined and it is unclear how they would be arranged; however, site-specific integration and enhancer/promoter trapping strategies can make use of endogenous enhancers to modify transgene expression, often resulting in unique expression patterns distinct from defined promoters (O'Brochta *et al*., 2012; Reid *et al*., 2018).

3.2.4   Chromatin structure and genomic position effects

Genes display complex and highly regulated profiles of expression that are dictated not only by local promoter, enhancer and silencer elements, but also by local chromatin structure, distal enhancers/silencers and epigenetic modifications that are, together, known as position effects. Indeed, the position of nucleosomes and dynamic epigenetic modifications to DNA and histones can affect accessibility of promoters to transcription factors, core transcriptional machinery and other DNA-binding proteins. These factors have been extensively studied in the fruit fly, but efforts to uncover these mechanisms in mosquitoes has begun only

Table 3.1. Promoters used to regulate transgene expression in mosquitoes. Sex and stage specificity are conserved in the core expression profile of transgenics unless indicated otherwise.

Tissue specificity of endogenous gene	Sex specificity	Stage specificity	Promoter	Parent species	Gene ID (upstream promoter region)	Species active	Core expression profile in transgenic insects	References
Ubiquitous	Male and Female	Ubiquitous	*Polyubiquitin*	*D. melanogaster*	FBgn0003943 (2 kb)	*Ae. aegypti, A. albimanus*	Broad expression in most somatic and germline tissues	Petra *et al*., 2002; Jasinskiene *et al*., 2007
				An. gambiae	AGAP001971 (5'-2005 bp, 3'-407 bp)	*An. gambiae*	Broad expression in most somatic and germline tissues	Adolfi *et al*. 2018
				Ae. aegypti	AAEL003877 (1,386 bp)	*Ae. aegypti*	Broad expression in most somatic and germline tissues	Travanty *et al*., 2004; Anderson *et al*., 2010; Li *et al*. 2017; Li *et al*. 2020
			Ribosomal Protein L40	*Ae. aegypti*	AAEL006511 (5' 421 bp)	*Ae. aegypti*	Ovary-, germline, thorax- and midgut-bias, L1 and adult bias	Anderson *et al*., 2010; Li *et al*. 2017; Li *et al*. 2020
				C. quinquefasciatus	CPIJ002413 (5'-3413 bp, 3'-865 bp)	*C. quinquefasciatus*	ND	Feng *et al*. 2021
			Actin 5C	*D. melanogaster*	FBgn0000042 (540 bp)	*An. stephensi, Ae. aegypti, An. gambiae, C. quinquefasciatus*	Posterior midgut and gastric caecae	Catteruccia *et al*., 2000; Pinkerton *et al*., 2000; Allen *et al*., 2001; Ren *et al*., 2008
				C. quinquefasciatus	CPIJ009808 (5'-7,368 bp, 3'-699 bp)	*C. quinquefasciatus*	ND	Feng *et al*. 2021

Early embryos	Male and Female	Embryos	KLC2	*Ae. aegypti*	AAEL011410 (1 kb)	*Ae. aegypti*	Male germline (pupae-adults) and early zygote	Hu and Tu, 2018
Ovary and embryos	None (embryo), Female specific (adult)	Embryos, pupae–adults, MD	bZip1	*Ae. aegypti*	AAEL009263 (5' 4,587 bp, 3'-150 bp from *A. stephensi B2* gene ASTEI09889)	*Ae. aegypti*	Female germline (pupae-adults) and early zygote	Kojin *et al*. 2020
Ovaries (female germline)	Female	L1–adults, MD	*Trunk*	*Ae. aegypti*	AAEL007584 (3,041 bp)	*Ae. aegypti*	Female germline	Akbari *et al*. 2014; Li *et al*. 2017; Li *et al*. 2020
		Pupae– adults, MD	*AAEL000923*	*Ae. aegypti*	AAEL010923 (2,934 bp)	*Ae. aegypti*	Female germline	Akbari *et al*. 2014; Li *et al*. 2017; Li *et al*. 2020
		Adults	*Vitellogenin Receptor*	*Ae. aegypti*	AAEL014223 (1.5 kb)	*Ae. aegypti*	Female germline	Cho *et al*., 2006
		L1–adults, MD	*4-nitro*	*Ae. aegypti*	AAEL007907 (2.5 kb)	*Ae. aegypti*	Female germline	Akbari *et al*., 2014
Testes (male germline)	Male	L3–adults	β2-tubulin (*B2*)	*An. gambiae*	AGAP008622 (1.4 kb)	*An. gambiae*, *An. stephensi*	Male germline	Catteruccia *et al*., 2005; Windbichler *et al*., 2008
				Ae. aegypti	DQ833526 (1 kb)	*Ae. aegypti*	Male germline	Smith *et al*., 2007
				C. capitata	931 bp	*C. capitata*	Male germline	Scolari *et al.,* 2008; Meccariello *et al*., 2021
		L1–adults, MD	*vasa-1*	*An. gambiae*	AGAP008578 (Vas1 -3,758 to -1,801)	*An. gambiae*	Male germline	Papathanos *et al*., 2009; Hammond *et al*., 2021

Continued

Table 3.1. Continued.

Tissue specificity of endogenous gene	Sex specificity	Stage specificity	Promoter	Parent species	Gene ID (upstream promoter region)	Species active	Core expression profile in transgenic insects	References
Gonads (male and female germline)	Strong female bias	Pupae– adults, MD	*Exuperantia (Exu)*	*Ae. aegypti*	AAEL010097 (3,193 bp)	*Ae. aegypti*	Female and male germline	Akbari *et al*., 2014
	Male	Adults		*An. gambiae*	AGAP007365 (5'-849 bp, 3'-1,173 bp)	*An. gambiae*	Male germline	Hammond *et al*., 2021
	Male and Female	Adults, no MD	Zero population growth *(zpg)*	*An. gambiae*	AGAP006241 (5'-1,074 bp, 3'-1,037 bp)	*An. gambiae*	Female and male germline	Hammond *et al*., 2021
		L1–adults, MD	*vasa-2*	*An. gambiae*	AGAP008578 (Vas2 2,291 to +1)	*An. gambiae, Ae. aegypti* (female specific)	Germline, MD, leaky in soma	Papathanos *et al*., 2009; Akbari *et al*., 2014; Hammond *et al*., 2021
				An. stephensi	ASTE003241 (5'-4,009 bp, 3'-1,014 bp)	*An. stephensi*	Germline, MD, leaky in soma	Gantz *et al*., 2015; Adolfi *et al*., 2020
				C. quinquefasciatus	CPIJ009286 (5'-2,414 bp, 3'-761 bp)	*C. quinquefasciatus*	Germline, MD, leaky in soma	Feng *et al*., 2021
		Adults, MD, PD	*nup-50*	*Ae. aegypti*	AAEL005635	*Ae. aegypti*	Germline, MD, PD	Li *et al*. 2017; Li *et al*. 2020
		Adults, MD	*Nanos (Nos)*	*An. stephensi*	(5'-3.8 kb, 3'-679)	*An. stephensi*	Germline, MD, and soma	Macias *et al*., 2017;
				An. gambiae	AGAP006098 (5'-2,092 bp, 3'-601 bp)	*An. gambiae*	Germline, MD	Meredith *et al*., 2013; Hammond *et al*., 2021; Carballar-Lejarazú *et al*., 2020

Tissue	Sex	Stage	Gene	Source	Accession	Species	Expression	Reference
				C. quinquefasciatus	CPIJ011551 (5'-4,174 bp, 3'-1,399 bp)	*C. quinquefasciatus*	Germline, MD	Feng *et al.*, 2021
	Female (putative)			*Ae. aegypti*	AAEL012107 (5'-1.6 kb, 3'-335)	*Ae. aegypti*	Germline (putatively female specific), MD	Adelman *et al.*, 2007
Fat body	Female	L1–adults	*Lipophorin (Lp)*	*An. gambiae*	AGAP001826 (1.6 kb)	*An. gambiae*	Fat body, midgut in L1	Volohonksy *et al.*, 2015
		Adults (PBM)	*Vitellogenin (Vg)*	*An. gambiae*	AGAP004203 (0.85 kb)	*An. stephensi, An. gambiae*	Fat body	Nirmala *et al.*, 2006; Chen *et al.*, 2007; Volohonksy *et al.*, 2015
				An. stephensi	DQ442990	*An. stephensi*	Fat body	Isaacs *et al.*, 2012
				Ae. aegypti	AAEL010434 (2.1 kb)	*Ae. aegypti*	Fat body	Kokoza *et al.*, 2001a, b; Isoe *et al.*, 2007
	Female bias	L4–young adults	*Hexamerin-1.2*	*Ae. (Ochlerotatus) atropalpus*	(–714 to –36)	*Ae. aegypti*	Fat body	Totten *et al.*, 2013
	Male and Female	L2–adults	*Anopheles Plasmodium-responsive Leucine-rich repeat 1C (APL1C)*	*An. gambiae*	AGAP007033 (2,050 bp)	*An. gambiae*	Fat body	Volohonsky *et al.*, 2017
Fat body, haemocytes	Male and Female	L2–adults	*Leucine-rich immune protein (Long) (LRIM1)*	*An. gambiae*	AGAP006348 (1,906 bp)	*An. gambiae*	Fat body	Volohonsky *et al.*, 2017
			Thioester-containing protein 1 (TEP1)	*An. gambiae*	AGAP010815 (3,103 bp, 268 bp minimal promoter)	*An. gambiae*	Fat body	Volohonsky *et al.*, 2017

Continued

Table 3.1. Continued.

Tissue specificity of endogenous gene	Sex specificity	Stage specificity	Promoter	Parent species	Gene ID (upstream promoter region)	Species active	Core expression profile in transgenic insects	References
Haemocytes	Male and Female	L4-adults	*Prophenoloxidase gene ppo6* (*ppo6*)	*An. gambiae*	AGAP004977 (1.6 kb)	*An. gambiae*	Haemocytes	Volohonksy *et al.* 2015; Volohonsky *et al.*, 2017
		Cells	*CEC1*	*An. gambiae*	AGAP000693	*An. gambiae*	*in vitro* assay only	Lombardo *et al.*, 2013
	Strong female bias	Adults, BMI	*Hemolectin*	*D. melanogaster*	FBgn0029167 (5'-1,160)	*An. gambiae*	Haemocytes	Pondeville *et al.* 2020
Midgut	Female	Pupae–adults (24 h BMI)	*Carboxypeptidase A*	*An. gambiae*	AGAP009593 (3.4 kb and in frame fusion)	*Ae. aegypti, An. stephensi, An. gambiae*	Midgut and ventral nerve cord, 24 h PBM (*A. aegypti*), 3–6 h PBM (*A. stephensi*)	Moreira *et al.*, 2000; Ito *et al.*, 2002; Meredith *et al.*, 2011; Isaacs *et al.*, 2012; Hoerrmann *et al.* 2021
				Ae. aegypti	AAEL010782 (1.4 kb)	*Ae. aegypti, An. gambiae*	Midgut	Moreira *et al.*, 2000; Kim *et al.*, 2004
			G12	*An. gambiae*	AGAP006718 (1.1 kb)	*An. stephensi, An. gambiae*	Midgut	Nolan *et al.*, 2011; Volohonsky *et al.* 2015
	Female bias	Adults (24–40 h BMI)	*Trypsin-1*	*An. gambiae*	AGAP008296 (1.1 kb)	*An. stephensi*	Variable/low level expression, midgut, BMI 6–48 h	Nolan *et al.*, 2011

Tissue	Sex	Stage	Gene	Source	Accession (size)	Tested species	Expression	References
		Adults (3 h BMI)	*Adult Peritrophic Matrix Protein 1*	*An. gambiae*	AGAP006795 (2.6 kb and in frame fusion)	*An. stephensi, A. fluviatilis*	Midgut	Abraham *et al*., 2005; Rodrigues *et al*., 2008; Hoerrmann *et al*. 2021
		Adults	*Alkaline phosphatase 2 (AP2)*	*An. gambiae*	AGAP006400 (in frame fusion)	*An. gambiae*	Midgut	Hoerrmann *et al*., 2021
Salivary gland	Female	Pupae–adults	*D7r4*	*An. gambiae*	AGAP008281 (0.9 kb)	*An. stephensi*	None, highly variable tissue expression	Lombardo *et al*., 2005
		Adults (BMI)	*Anopheline Antiplatelet Protein (AAP)*	*An. stephensi*	ASTM015525 (1.7 kb)	*An. stephensi*	Salivary gland	Yoshida and Watanabe, 2006
	Female bias	Adults	*Apyrase*	*An. gambiae*	AGAP011971, (2.4 kb)	*An. gambiae, An. stephensi*	Low level, poorly regulated, salivary gland proximal-lateral lobe bias	Lombardo *et al*., 2000, 2005, 2009
				Ae. aegypti	AAEL006347 (1.5 kb)	*Ae. aegypti*	Salivary gland	Coates *et al*., 1999
	Male and Female	Adults	*Maltase-like I*	*Ae. aegypti*	AAEL000392 (1.5 kb)	*Ae. aegypti*	Salivary gland	Coates *et al*., 1999
Indirect flight muscle	Male and Female	Pupae–adults	*Actin88F*	*D. melanogaster*	FBgn0000047	*C. quinquefasciatus*	Flight muscle	Allen and Christensen, 2004
	Female bias (+ alternative splicing)	L4–adults	*Actin-4*	*Ae. aegypti*	AAEL001951 (3.3 kb)	*Ae. aegypti, An. stephensi, Ae. albopictus*	Indirect flight muscle	Fu *et al*., 2010; Labbé *et al*., 2012; Marinotti *et al*., 2013
				Ae. albopictus	9JN709493.1 (0.7 kb)	*A. albopictus*	Indirect flight muscle	Labbé *et al*., 2012
				An. stephensi	ASTM009772 (1 kb)	*An. stephensi*	Indirect flight muscle	Marinotti *et al*., 2013

Continued

Table 3.1. Continued.

Tissue specificity of endogenous gene	Sex specificity	Stage specificity	Promoter	Parent species	Gene ID (upstream promoter region)	Species active	Core expression profile in transgenic insects	References
Chemosensory appendages and brain	Male and Female	Late larvae–adults	Odorant receptor co-receptor (ORCO)	An. gambiae	AGAP002560 (9,312 bp)	An. gambiae	Olfactory sensory neurons within chemosensory appendages	Riabinina et al., 2016
				Ae. aegypti	AAEL005776 (in-frame fusion)	Ae. aegypti	Olfactory sensory neurons within chemosensory appendages	Shankar et al., 2020
			Ionotropic Receptor co-receptor IR8a	Ae. aegypti	AAEL002922 (in-frame fusion)	Ae. aegypti	Olfactory sensory neurons within chemosensory appendages	Shankar et al., 2020
			Gustatory receptor 1 (Gr1)	Ae. aegypti	AAEL002380 (in-frame fusion)	Ae. aegypti	Olfactory sensory neurons within chemosensory appendages	Shankar et al., 2020
			ppk301	Ae. aegypti	in-frame fusion	Ae. aegypti	Sensory neurons within chemosensory appendages	Matthews et al., 2019
			Ammonium transporter (Amt)	An. gambiae	AGAP003989 (1 kb/3 kb)	An. gambiae	Neuronal and auxiliary cells in antennal tissue	Ye et al., 2020
Pan-neuronal	Male and Female	Early larvae–adults	Synaptotagmin-1 (Syt1)	Ae. aegypti	AAEL000704	Ae. aegypti	Pan-neuronal	Zhao et al., 2021
			Bruchpilot (Brp)	Ae. aegypti	AAEL018153	Ae. aegypti	Pan-neuronal	Zhao et al., 2021
Central nervous system	Male and Female	Embryo, other stages N/A	γ-aminobutyric acid receptor (GABAR)	Ae. aegypti	AAEL008354 (2.5 kb)	Ae. aegypti	Embryo (other stages N/A)	Shotkoski et al., 1996

Eyes and nervous system	Male and Female	L1–adults	*3xP3*	*D. melanogaster*	Synthetic	Most insects, including *An. gambiae*, *An. stephensi*, *Ae. aegypti*, *Ae. albopictus*	Photoreceptor cells, brain, anal papillae	Sheng *et al.*, 1997; Horn *et al.*, 2000; Kokoza *et al.*, 2001a; Ito *et al.*, 2002; Groth *et al.*, 2004; Catteruccia *et al.*, 2005; Labbé *et al.*, 2010
Midgut and gastric caeca	Male and Female	L1–Adults	*Actin-5c (Act5c)*	*D. melanogaster*	FBgn0000042 (540 bp)	*An. gambiae, A. funestus, Ae. aegypti*	Midgut and gastric caeca	Catteruccia *et al.*, 2000; Quinn *et al.*, 2021; Kandul *et al.*, 2021
				C. quinquefasciatus	CPIJ009808 (5'-7,368 bp, 3'-699 bp)	*C. quinquefasciatus*	Midgut and gastric caeca	Feng *et al.*, 2021
Ubiquitous	Male and Female	Ubiquitous	*U6 promoter*	*An. gambiae, Ae. aegypti, C. quinquefasciatus*	Various	*An. gambiae, Ae. aegypti, C. quinquefasciatus*	Neuronal and auxiliary cells in antennal tissue	Konet *et al.*, 2007; Dong *et al.* 2015; Hammond *et al.* 2016; Anderson *et al.*, 2020; Feng *et al.*, 2021

Abbreviations: BMI, blood meal induced expression; L, larva; MD, maternally deposited transcript; ND, no data; PBM, post-blood meal specific expression; PD, paternally deposited transcript

recently (reviewed in Sharakhov and Sharakhova, 2015; Lezcano *et al*., 2020). Genome-wide profiling of chromatin accessibility has been used to reveal *cis*-regulatory elements in the genomes of *Anopheles gambiae* (Perez-Zamorano *et al*., 2017) and *Aedes aegypti* (Behura *et al*., 2016; Mysore *et al*., 2018), some of which were shown to be active in *Drosophila* (Mysore *et al*., 2018). Exciting new research points to specific instances where differences in chromatin accessibility are associated with tissue-specific differences in gene expression, such as the immune protein *lrim1* in *An. gambiae* for which strong salivary gland expression is directly correlated with tissue-specific chromatin state (Ruiz *et al*., 2021).

3.3   Post-transcriptional and Translational Control

Once an mRNA transcript is produced, its stability, localization and cell-type specific splicing and translation are controlled by interactions with RNA-binding proteins and regulatory RNAs, such as Piwi-interacting RNA (piRNA), microRNA (miRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA) and long non-coding RNA (lncRNA). These processes allow a fine-tuning of the core expression dictated by the promoter and other *cis*-regulatory elements.

3.3.1   Untranslated regions and introns

Untranslated regions (UTRs) are the transcribed sequences immediately upstream or downstream of a coding DNA sequence (CDS) that are not translated alongside the primary CDS. Their primary function is to recruit RNA-binding proteins that mediate translation, transcript stability and transcript localization, which can be further fine-tuned by physical interaction between 5′ and 3′ UTRs.

An often-overlooked feature of the UTR, particularly the 3′ UTR, is its ability to restrict translation to specific cell types through interactions with suppressors – a layer of regulation that cannot be achieved with promoters alone. The best characterized examples are the maternal effect genes *nanos* and *zpg* that are broadly deposited into the embryo, where signals on the UTR mediate both mRNA trafficking to the embryonic germ plasm and germline restricted translation (Tazuke *et al*., 2002; Rangan *et al*., 2009).

Specific sequences within the UTRs, known as translational enhancers, have been shown to dramatically enhance transcript and protein levels in insects and can be included in transgenic constructs to boost protein expression (Pfeiffer *et al*., 2010). Conversely, some UTRs carry upstream open reading frames (uORFs) that effectively suppress translation of their cognate mRNA, and can be artificially included to dampen transgene expression (Abraham *et al*., 2005; Southall *et al*., 2013). Indeed, the term UTR can be a misnomer, due to the presence of uORFs.

In addition to UTRs, there is a growing appreciation that introns can boost transcript levels, through both splicing-dependent and splicing-independent mechanisms, affecting the rate of transcription, nuclear export and transcript stability. This phenomenon is known as 'intron-mediated enhancement' and is reviewed in Shaul (2017). Additionally, there is increasing evidence of promoter-like interactions between intron transcription factors that can also affect mRNA levels, though the evidence for this is currently strongest in plants (Gallegos and Rose, 2017).

3.3.2   Regulatory RNAs

Non-coding RNAs (ncRNAs) such as piRNAs, miRNAs and lncRNAs participate in a wide range of regulatory activities, such as epigenetic regulation, transcript silencing and splicing (Ma *et al*., 2021). Small non-coding RNAs play an important role in modulating gene expression by targeting mRNA transcripts, usually at the 3′ UTR, for degradation by Argonaute and Piwi-protein dependent pathways. In mosquitoes, small

non-coding RNAs are expressed in a wide range of tissues, most abundant in the midgut and least abundant in the ovaries (Bryant *et al*., 2019, 2020), where they carry out essential tasks such as targeting transposon and virus RNA for degradation (Ma *et al*., 2021). Though ncRNAs are least abundant in germline tissues, piRNAs play an essential and conserved role in gametogenesis, underpinned by their ability to silence and prevent the remobilization of transposable elements in the germline cells (Vagin *et al*., 2006; Brennecke *et al*., 2007). Additionally, there is evidence of this regulatory role being co-opted into the control of endogenous mRNAs essential for developmental transitions and sex determination (Tang *et al*., 2018). lncRNAs are ncRNA molecules exceeding 200 nt in length that participate in both transcript and epigenetic regulation, with potentially important roles in insect immunity and germline maintenance (Satyavathi *et al*., 2017). Both *Aedes* and *Anopheles* mosquitoes encode almost 3000 lncRNAs, some of which show strong sex-specific biases that are important for fertility (Jenkins *et al*., 2015; Xu *et al*., 2019) and whose role can be dependent on further interactions with other ncRNAs such as piRNAs (Betting *et al*., 2021).

3.3.3 Splicing

Intron retention and alternative splicing create an incredible diversity of the transcriptome, with transcript isoforms outnumbering genes by 17-fold in *Drosophila melanogaster* (Brown *et al*., 2014). More than 90% of genes are alternatively spliced, with approximately 1% being processed into hundreds of transcripts that can direct cell-type-specific diversity. The extreme diversity of the transcriptome is most apparent in neuronal and embryonic tissues, whereas only several hundred genes show a sex bias – often limited to the gonads (Brown *et al*., 2014). This is also the case for mosquitoes, in which 10% of alternatively spliced genes have a strong tissue bias, 1% show complete tissue specificity and just a handful

show sex-specific splicing (Sreenivasamurthy *et al*., 2017). Indeed, sex determination appears to be the only biological process that is controlled by sex-specific splicing, initiated by a splice cascade of core sex determination genes including *doublesex* (*dsx*), *fruitless* (*fru*) and *transformer* (*tra*), reviewed in Wexler *et al*. (2019)(see Arien *et al*., Chapter 10, this volume). Sex-specific isoforms of these genes encode transcription factors or splicing factors that establish developmental programming in all insects studied thus far and recent research suggests that cell-type-specific enhancers mediate further control by enforcing tissue-specific splicing (Rice *et al*., 2019).

3.3.4 Translational control

Translational control can affect gene expression in myriad ways, including total levels of expression, spatio-temporal specificity, protein stability and intracellular or extracellular localization. These features can be encoded by the protein itself, such as peptide signals conferring localization to the nucleus, cytosol, membrane and export out of the cell – which is important for immune and salivary gland proteins that can affect parasite development and transmission. Translational control can also be dictated by the mRNA transcript itself, for example by the presence of motifs or structures in the UTR (some referred to above) that affect the efficiency of initiation of translation by the ribosome machinery, or by codon biases. All organisms show a species-specific bias in codon usage that appears to fine-tune translational efficiency of an mRNA transcript, including surprising differences between *Aedes* and *Anopheles* mosquitoes (Behura and Severson, 2011), reviewed in Behura and Severson (2012). In insects, the greatest bias is seen in highly expressed housekeeping genes and whilst these authors would generally not advise altering codon bias between insect species when expressing a transgene, prokaryotic genes such as Cas9 are typically codon-optimized for expression in eukaryotes.

3.4 The Basic Genetic Construct

The minimal requirements for reliable and specific transgene expression can be described as the expression cassette, comprising a coding sequence (CDS), promoter (containing *cis*-regulatory elements and the 5′ untranslated region), and a terminator (containing the 3′ untranslated region and signals to terminate transcription by the RNA polymerase). For the purpose of transgenesis, the promoter and terminator sequences are usually defined as the entire regulatory sequences upstream of the start codon and downstream of the stop codon, respectively. Together, they are the most important elements controlling specificity of transgene expression. Several expression cassettes designed to express transgenes and markers are incorporated into the basic genetic construct, which must also carry sequences designed to mediate integration into the insect genome, varying according to the method used to achieve transgene integration – typically either inverted repeats (IRs) used by transposases (see O'Brochta, Chapter 1, this volume) , attachment sites such as attP used by integrases, or homology arms (HA) used for CRISPR-mediated homology-directed

repair (HDR) (see Ahmed and Wimmer, Chapter 5, this volume) (Fig. 3.1).

3.5 Sex-Specific Gene Expression

Sex-specific expression is crucial to the development of several genetic control strategies, such as the sterile insect technique (SIT) (see Scott *et al.*, Chapter 17, this volume), gene drive and the general approach of expressing anti-parasitic effectors in female mosquitoes. It is also useful as a tool to investigate sex determination and sexual development. Fortunately, strategies to achieve sex-specific expression vary more widely than for stage or tissue specificity, due to sexual dimorphisms at the level of chromosomes and transcriptional programmes that can be exploited to achieve expression that is restricted to a single sex.

3.5.1 Targeting chromosomes

In insects with an XY system for sex determination, the heterogametic sex is usually the male and insertion of a transgene into
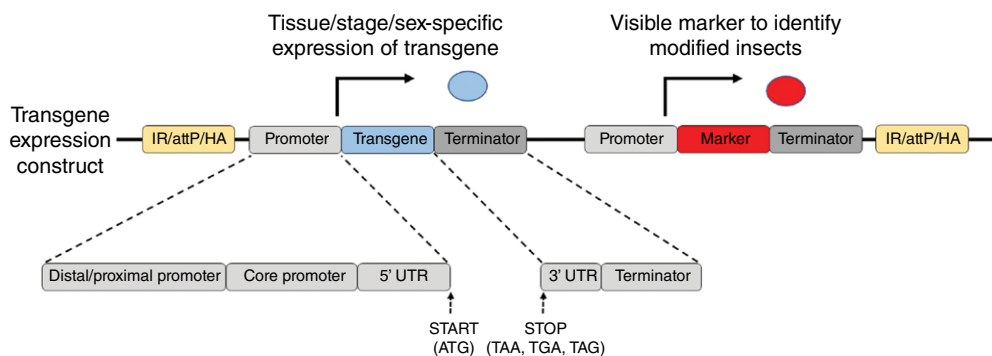
Fig. 3.1. The basic genetic construct. Transgenic constructs designed for expression in insects must contain sequences that allow its integration into the genome (yellow), such as attP sequences used by φC31 integrase, inverted repeats (IRs) used by transposases, or homology arms (HAs) used in CRISPR-mediated HDR. The transgene (blue) is typically placed under transcriptional control of a promoter containing distal, proximal and core promoter regulatory sequences, as well as a 5′ UTR, and followed by a terminator that contains a 3′ UTR. To help identify transgenic insects, a second expression cassette should be included that can drive strong and unambiguous expression of a visible marker – such as green fluorescent protein (GFP) or another fluorescent protein.

the Y chromosome will confer male specificity. This approach has proven successful for *An. gambiae*, either by using CRISPR-mediated HDR (Bernardini *et al*., 2018) or secondary modification of an attP site mediated by φC31 integrase (Bernardini *et al*., 2014). Unfortunately, the Y chromosome is highly repetitive and AT-rich, making it difficult to sequence and difficult to design an effective CRISPR-Cas gRNA against. Moreover, large regions of the Y chromosome are heterochromatic and can be subject to variegated position effects.

Many other insect species do not have XY systems for sex determination and may rely upon X:A ratio, or the expression of an autosome-linked male determining factor such as *nix* in *Ae. aegypti* (Hall *et al*., 2015). Whilst promoters from male-determining factors do not tend to drive male-specific expression when inserted elsewhere in the genome (Criscione *et al*., 2013; Meccariello *et al*., 2019; Aryan *et al*., 2020), it may be possible by placing transgenes under control of their original promoters and inserting at or close to the native sex-determining locus to take advantage of local enhancers.

3.5.2 Sex-specific splicing

Sex-specific splicing can also be exploited for either male- or female-specific transgene expression; for example, transgenes placed inside synthetic exons designed to replicate sex-specific splicing of the genes *doublesex* (female-specific exon) and *fruitless* (male-specific exon) have been successfully used in a range of insect species (Phuc *et al*., 2007; Magnusson *et al*., 2011; Jin *et al*., 2013). This strategy is likely to be successful in most insect species, because *doublesex* and *fruitless* are highly conserved genes in the insect sex determination pathway and show similar splicing patterns across a range of species (Salvemini *et al*., 2010, 2011; Price *et al*., 2015). This is in stark contrast to other members of the pathway, such as the *Drosophila* gene *sex-lethal,* which appears to play no role in sex determination in nondrosophilid insects. Unfortunately, very few

genes show complete sex-specificity between splice variants (Telonis-Scott *et al*., 2009) and there may be as few as about 30–40 genes showing complete sex-specificity within the *Anopheles* genus (Papa *et al*., 2017). Further, the genetic engineering required to faithfully recapitulate, or hijack, sex-specific splicing of endogenous genes may be more complex than simply testing the upstream regions of sex-specific genes to see if they can confer sex-specific transgenic expression.

3.5.3 Sex-specific promoters

Many genes are naturally sex-specific and their promoters have been used to drive sex-specific expression in a wide range of insects (Table 3.1). These include genes involved in the development or function of sex-specific tissues such as the ovaries, testes and their accessory organs, but also sexually dimorphic tissues such as the midgut, indirect flight muscle, claspers, head, chemosensory appendages and the fat body. The male-specific *β2-tubulin* promoter is active in diverse insect species (Catteruccia *et al*., 2005; Scali *et al*., 2005; Smith *et al*., 2007) and is an excellent candidate for others, because it is highly conserved, expressed at high levels and tightly restricted to male germline cells undergoing spermatogenesis. Another germline-specific promoter, *vasa-1*, is expressed only in males in *An. gambiae*, but can be modified to express in both sexes by inclusion of an intron from the 5′ UTR, known as the *vasa-2* promoter (Papathanos *et al*., 2009). Equivalent female-germline-specific genes have yet to be widely tested, but there are several promising candidates, such as the ovary-specific *trunk* promoter, which is expressed in nurse cells but maternally deposited into both male and female embryos (Akbari *et al*., 2014). Most genes expressed in the female germline are deposited into embryo, which may be a desirable or undesirable feature, depending on the eventual application. Sex-specific genes expressed in sexually dimorphic somatic tissues do not have this risk and can be

identified by mining transcriptomics data. These datasets cannot be used to guarantee that expression is exclusive to one sex and so candidates should be chosen on the basis of a known or putative role in a sex-specific process. For haematophagous insects, such promoters have been isolated from genes involved in female-specific processes such as yolk protein synthesis in the fat body (Kokoza *et al*., 2001a,b; Nirmala *et al*., 2006; Isaacs *et al*., 2012; Volohonsky *et al*., 2015), ovary development (Papathanos *et al*., 2009) and bloodmeal digestion (Moreira *et al*., 2000; Nolan *et al*., 2011). Similar advances have been made in the identification of promoters in the medfly *Ceratitis capitata*, including the two fat-body male-specific serum protein (MSSP) promoters (MSSP-α2 and MSSP-β2), which may prove useful for SIT (Komitopoulou *et al*., 2004).

3.6   Tissue-Specific Gene Expression

Tissue specificity is the most commonly sought control for transgene expression, as it allows full flexibility to manipulate a wide array of biological processes, including those relevant for parasite transmission, behaviour and gene drive.

3.6.1   Targeting tissues relevant for parasite transmission

Mosquitoes and other haematophagous insects require a bloodmeal to reproduce and can transmit viral, apicomplexan, filarial and trypanosome parasites to humans as a result. Parasites must encounter several insect tissues as they mature to their infective stage. Efforts to understand and block host–parasite interactions depend upon expression of effector molecules in these tissues and may include a requirement for bloodmeal-induced expression to coincide with parasite acquisition. To this end, promoters have been tested that can drive expression in the salivary glands (Coates *et al*., 1999;

Lombardo *et al*., 2000, 2005, 2009; Yoshida and Watanabe, 2006), midgut (Moreira *et al*., 2000; Ito *et al*., 2002; Kim *et al*., 2004; Abraham *et al*., 2005; Rodrigues *et al*., 2008; Meredith *et al*., 2011; Nolan *et al*., 2011; Isaacs *et al*., 2012; Volohonsky *et al*., 2015, 2017), fat body (Kokoza *et al*., 2001a,b; Nirmala *et al*., 2006; Chen *et al*., 2007; Isoe *et al*., 2007; Isaacs *et al*., 2012; Totten *et al*., 2013; Volohonsky *et al*., 2015; Hoermann *et al*., 2021) and immune cells called haemocytes (Lombardo *et al*., 2013; Volohonsky *et al*., 2015, 2017; Pondeville *et al*., 2020). Many of these have been used to express anti-pathogen effectors in the mosquito and recent efforts have incorporated these expression cassettes as a cargo/payload into gene drives designed to modify natural populations with the aim of reducing parasite transmission (Gantz *et al*., 2015; Adolfi *et al*., 2020; Carballar-Lejarazu *et al*., 2020) (see Bottino-Rojas and James, Chapter 11; and Franz, Chapter 22, this volume).

3.6.2   Targeting germline expression for gene drives

CRISPR-based homing gene drives designed to spread by homing require expression of Cas9 in the germline to induce HDR and this can be in both sexes or sex-specific, depending on the strategy. First-generation gene drives made use of the *vasa* promoter to drive high levels of Cas9 activity in the male and female germline, which resulted in high levels of homing (Gantz *et al*., 2015; Hammond *et al*., 2016). Unfortunately, high levels of maternal deposition in the embryo, where the mutagenic process of non-homologous end joining is the predominant repair pathway, led to the creation of drive-resistant mutations at the Cas9 target site (Hammond and Galizi, 2017; Hammond *et al*., 2021). Furthermore, maternal deposition combined with leaky somatic expression can reduce fitness by disrupting target genes in non-target tissues where the gene is required (Gantz *et al*., 2015; Hammond *et al*., 2016). To address these issues, several novel germline promoters have been tested that

show varying levels of germline expression and maternal/paternal deposition (Akbari *et al.*, 2014; Carballar-Lejarazu *et al.*, 2020; Li *et al.*, 2020; Hammond *et al.*, 2021). Of these, all appear to drive high levels of maternal deposition except for *zpg*, which can promote high levels of homing in the male and female germline of *Anopheles* mosquitoes without inducing embryonic end joining (Hammond *et al.*, 2021), though the suitability of this promoter in other insects remains to be seen. In *Ae. aegypti*, the *nup-50* promoter induces both maternal and paternal deposition but can also drive high levels of germline homing in both sexes (Li *et al.*, 2020).

3.6.3 Targeting expression in chemosensory neurons

Mosquitoes and other vector species use cues from their environment to identify potential hosts, oviposition sites, mates and other sources of food. Host-seeking behaviour is driven primarily by olfactory cues detected on sensory neurons throughout the chemosensory appendages – the antennae, maxillary palps and labella. Several hundred receptors mediate odour-specific responses, but it is unknown which receptors and odours are most important for host-seeking, mating and other epidemiologically relevant behaviours. This is particularly important for anthropophilic vector species such as *Ae. aegypti* and *An. gambiae,* whose extreme preference for humans over other sources of blood makes them uniquely important vectors of human disease. Fortunately, species-specific behaviours can also be targeted for vector control, such as attractive odour-baited traps and insect repellents.

To dissect and potentially disrupt these molecular pathways, researchers have created complex systems to express transgenes in broad subsets of neurons by using *cis*-regulatory elements from conserved olfactory co-receptor genes. Unlike other tissues, neuronal promoters are particularly susceptible to position effects and tend to drive very low levels of expression that may be insufficient to visualize or manipulate neuronal tissues. Binary expression systems (see section 3.9.8 below and Schetelig *et al.*, Chapter 2, this volume) such as Gal4-UAS (Brand and Perrimon, 1993) and the Q-system (Potter *et al.*, 2010) can be used to ramp up levels of expression with sex-, tissue- and stage-specificity, and have already revolutionized efforts to modify chemosensory neurons (Riabinina *et al.*, 2016). To further mitigate against position effects, several groups have targeted in-frame fusions of QF2 to endogenous neuronal genes in *Aedes* mosquitoes, allowing expression of activity-dependent sensors such as GCaMP and CaMPARI that can be used to monitor cell-specific responses to odours and other stimuli (Matthews *et al.*, 2019; Shankar *et al.*, 2020; Ye *et al.*, 2020; Zhao *et al.*, 2021).

3.7 Stage-Specific Gene Expression

As insects progress through developmental stages, widespread shifts in the transcriptome direct metamorphosis and responses to changing environmental conditions, behaviours and infection.

3.7.1 Targeting developmental stages

Most insects encounter dramatic metamorphoses throughout their development that can include eggs, larvae, pupae and adults. A plethora of transcriptomics studies have identified the changing landscape of gene expression throughout development in mosquitoes (Table 3.2), yet very few promoters have been isolated to drive specific expression in non-adult stages. To our knowledge just two zygotic promoters have been adapted for transgene expression in mosquitoes: *KCL2* (Hu and Tu, 2018) and *bZip1* (Kojin *et al.*, 2020), with only *KCL2* restricted to the embryos. Targeting zygotic or late-larval stages is important for the development of novel genetic control strategies, such as killer–rescue and adaption thereof (Hu and Tu, 2018; Webster *et al.*, 2020). Identification of such promoters is not simple, as only

Table 3.2. Studies investigating sex-, tissue- and stage-specific gene expression in *An. gambiae* and *Ae. aegypti*.

Stage/condition	Relevant studies in *Anopheles gambiae*	Relevant studies in *Aedes aegypti*
Development egg to adult	Marinotti *et al.*, 2005; Koutsos *et al.*, 2007; Neira Oviedo *et al.*, 2008; Goltsev *et al.*, 2009; Cook and Sinkins, 2010	Caragata *et al.*, 2011; Neira-Oviedo *et al.*, 2011; Biedler *et al.*, 2012; Akbari *et al.*, 2013; Harker *et al.*, 2013; Tomchaney *et al.*, 2014; Li *et al.*, 2017; Hu and Tu; 2018; Tallon *et al.*, 2019)
Plasmodium/dengue infection	Vlachou *et al.*, 2005; Pinto *et al.*, 2009; Mendes *et al.*, 2011	Behura *et al.*, 2011; Etebari *et al.*, 2015; Anglero-Rodriguez *et al.*, 2017; Raquin *et al.*, 2017
Bloodmeal	Marinotti *et al.*, 2005; Vannini *et al.*, 2014	Dissanayake *et al.*, 2010; Bonizzoni *et al.*, 2011; Raquin *et al.*, 2017
Circadian rhythm	Rund *et al.*, 2011	Leming *et al.*, 2014
Desiccation stress	Wang *et al.*, 2011	
Mating	Rogers *et al.*, 2008; Gabrieli *et al.*, 2014	Pascini and Martins, 2017
Insecticide	Isaacs *et al.*, 2018	Poupardin *et al.*, 2012; Riaz *et al.*, 2013; Maïga *et al.*, 2014; Faucon *et al.*, 2017
Tissues		
Male reproductive organs	Baker *et al.*, 2011; Shaw *et al.*, 2014; Cassone *et al.*, 2017; Papa *et al.*, 2017; Izquierdo *et al.*, 2019	Akbari *et al.*, 2013
Female reproductive organs	Baker *et al.*, 2011; Gabrieli *et al.*, 2014; Papa *et al.*, 2017; Bryant *et al.*, 2020	Akbari *et al.*, 2013; Pascini and Martins; 2017
Chemosensory appendages / brain	Pitts *et al.*, 2011; Rinker *et al.*, 2013; Hodges *et al.*, 2014	McBride *et al.*, 2014; Matthews *et al.*, 2016; Tallon *et al.*, 2019
Midgut	Neira Oviedo *et al.*, 2008; Baker *et al.*, 2011; Gomez-Diaz *et al.*, 2014; Bryant *et al.*, 2020	Akbari *et al.*, 2013; Raquin *et al.*, 2017
Salivary gland	Baker *et al.*, 2011; Gomez-Diaz *et al.*, 2014	Akbari *et al.*, 2013; Ribeiro *et al.*, 2016
Haemocyte	Pinto *et al.*, 2009	Choi *et al.*, 2012
Malpighian tubules	Baker *et al.*, 2011	Li *et al.*, 2017
Brain and antennae	Rinker *et al.*, 2013	Tomchaney *et al.*, 2014; Matthews *et al.*, 2016
Sex		
Male vs female	Baker *et al.*, 2011	Dissanayake *et al.*, 2010; Akbari *et al.*, 2013; Jiang *et al.*, 2015

18% of transcripts in the fly embryo are zygotically expressed, with the remainder maternally derived (Lott *et al.*, 2011). Whilst embryonic time-course transcriptomics can reveal such genes (Goltsev *et al.*, 2009; Biedler *et al.*, 2012), new studies aimed at single-cell RNA-seq may help in this regard and several studies have identified a number of genes exclusive to larval and pupal stages (Koutsos *et al.*, 2007; Akbari *et al.*, 2013; Harker *et al.*, 2013).

3.7.2 Targeting environmental, circadian and behavioural conditions

Adult insects engage in complex behaviours, many of which are relevant for disease transmission such as host-seeking, mating, insecticide avoidance and resting behaviours, which are in turn affected by endogenous cues such as circadian rhythm, bloodmeal ingestion and infection status. Effector transgenes expressed during these conditions

can be used to target pathogens, induce mortality, or modify mosquito behaviour to interrupt disease transmission. Likewise, investigation and expression of immune genes after infection can help in understanding and boosting natural immunity. To this end, researchers have investigated global transcriptome changes in response to a wide range of endogenous and exogenous cues, such as circadian rhythm (Rund *et al.*, 2011), *Plasmodium* infection (Vlachou *et al.*, 2005; Mendes *et al.*, 2011), bloodmeal (Marinotti *et al.*, 2005; Dissanayake *et al.*, 2010; Bonizzoni *et al.*, 2011), *Wolbachia* (Kambris *et al.*, 2009), desiccation stress (Wang *et al.*, 2011) and mating (Rogers *et al.*, 2008; Gabrieli *et al.*, 2014).

3.8 Design of Expression Systems for Sex-, Tissue- and Stage-Specific Transgene Expression

The section below discusses common design criteria to achieve sex-, tissue- and stage-specific expression in insects and lists promoters previously characterized in mosquitoes. We draw upon the experience of ourselves and others to give guidelines on how to choose the most appropriate system for expression and how best to design such a system. This includes advice on promoter length and when to include UTRs, introns, translational enhancers and insulator elements. We also describe strategies to mitigate against position effects by use of docking sites, CRISPR-mediated HDR, in-frame fusions and binary expression systems.

3.9 Mining Transcriptomics Data for Promoter Design

Though the list of characterized promoters is growing rapidly, there remains a large subset of tissues and stages wherein promoters that can reliably drive expression are not yet available. The first port of call when characterizing a new promoter is to identify endogenous genes with the desired expression profile. Fortunately, a plethora of transcriptomics studies have generated datasets that can be mined for such genes, whether it be developmental, tissue/sex-specific, or conditionally induced (Table 3.2).

Datasets such as these have been collated for a large number of vector species and compiled into VectorBase (http://www.vectorbase.org), an online bioinformatics resource and repository for vector genomes (Giraldo-Calderon *et al.*, 2015). VectorBase allows the user to build complex searches via workspace tools that can be used to identify broad groups of genes with similar expression profiles, or used to filter specific expression characteristics based on RNAseq and microarray datasets. Likewise, transcriptomic databases that catalogue sexually dimorphic or tissue-specific expression have been developed for *A. gambiae* (MozAtlas: http://www.tissue-atlas.org) and *D. melanogaster* (FlyBase: http://flybase.org, and Flyatlas2: http://flyatlas.gla.ac.uk/FlyAtlas2) (Drysdale and FlyBase Consortium, 2008; Baker *et al.*, 2011; Leader *et al.*, 2018).

3.9.1 Limiting the promoter length

In many insects, including *Anopheles* mosquitoes, the most important regulatory elements are generally captured within 3 kb upstream of the translational start site and 1 kb downstream of the CDS, and should include both introns and UTRs. Unfortunately, this is not always the case and can be particularly problematic for species with largely expanded genomes, such as *Ae. aegypti*, where long introns spanning 20–50 kb can sit within 5′ UTRs. To remedy this, we recommend combining RNAseq data with whole-genome sequencing to identify endogenous genes with a desirable expression profile whose nearest upstream gene is no more than 2–5 kb away, so that the most important *cis*-regulatory elements can be captured within a shorter promoter region. Alternatively, promoters from closely related species may effectively recapitulate the desired expression profile. For example, promoters from *Anopheles* are generally shorter than those of *Aedes* and maintain their specificity

across the two species (see Table 3.1). New strategies to uncover regulatory motifs may help inform promoter design, and limit the overall length, by combining next-generation sequencing technologies with strategies to probe chromatin state and putative transcription-factor binding such as FAIRE-seq, ATAC-seq and ChIP-seq (Behura *et al.*, 2016; Ruiz *et al.*, 2021).

3.9.2   The importance of the UTR

The most common strategy for transgene expression makes use of an endogenous promoter (and its 5′ UTR) combined with a viral or non-tissue-specific 3′ UTR/terminator such as the SV40 or hsp70 terminators. Whilst usually sufficient to drive strong expression in the tissue of interest, the strategy can be leaky, often failing to limit spatio-temporal expression to that of the endogenous gene. Two important but often overlooked features of UTRs is their ability to localize mRNA transcripts and to restrict translation. This is more commonly a feature of the 3′ UTR and can help restrict expression in space and time. It is particularly essential for the specific activity of maternal effect genes, many of which are broadly deposited into the developing oocyte but transported to specific regions where their translation is carefully delimited. This has proved to be an important consideration in the development of homing-based gene drives for which tightly controlled expression is essential to direct Cas9-mediated HDR in the germline whilst avoiding somatic mutations that can reduce fitness or generate resistance to drive. Indeed, *vasa*, *zpg* and *nanos* transcripts are germline expressed but also maternally deposited into the embryo (Goltsev *et al.*, 2009; Papa *et al.*, 2017). Both *nanos* and *zpg* contain regulatory elements on the UTRs that can restrict translation of their endogenous protein to the embryonic germplasm (Tazuke *et al.*, 2002; Rangan *et al.*, 2009) and evidence in *An. gambiae* would suggest that regulatory sequences flanking *zpg* and *nanos*, but not *vasa*, are sufficient to prevent substantial protein expression in the embryo (Hammond *et al.*, 2021).

3.9.3   Boosting levels of expression

The simplest strategy to achieve high levels of transgene expression is to identify endogenous genes with the highest levels of expression in that target tissue by analysing available transcriptomics data. Indeed, this strategy proved effective in the identification of genes expressed at high levels in the male (Catteruccia *et al.*, 2005) and female germline of mosquitoes (Akbari *et al.*, 2014; Hammond *et al.*, 2021), but it is sometimes important to boost expression beyond what can be achieved using endogenous promoters by including translational enhancers, UTRs and introns, or by optimizing codon usage. Alternatively, transgenes can be placed within a binary expression system (see section 3.9.4 below).

Translational enhancers are perhaps one of the simplest modifications to an expression cassette that can substantially increase levels of protein expression. Several of these sequences derived from plant and insect viruses have been demonstrated to increase levels of expression by as much as 20-fold in *Drosophila* (Pfeiffer *et al.*, 2012). Although the most commonly used 3′ UTR is derived from the simian virus 40 (SV40), the p10 3′ UTR from *Autographa californica* nucleopolyhedrovirus can increase expression by tenfold when used on its own, or 20-fold when used in combination with the synthetic 5′ UTR element Syn21 – itself conferring a 7.5-fold increase in expression when used alone (Pfeiffer *et al.*, 2012).

Introns have also been demonstrated to exert a modest improvement in transgene expression levels in flies (Pfeiffer *et al.*, 2010), most likely by affecting the rate of transcription, nuclear export and transcript stability. The strategy has yet to be applied to non-drosophilid insects as a means to enhance gene expression; however, introns have been used recently in *Anopheles* mosquitoes to place entire expression cassettes within the introns of other genes (Hoermann *et al.*, 2021).

Nevertheless, introns are not simple to include in expression cassettes, as their effects on expression and splicing may be tissue specific, and the mechanism underlying this effect varies greatly between introns (reviewed in Shaul, 2017).

3.9.4 Dampening levels of expression

Most promoters used for expression in non-drosophilid insects have been chosen for their ability to drive strong expression, but some transgenes may be toxic, or exhibit undesirable effects when expressed at high levels. Experimenters wishing to reduce expression levels can take several approaches to this end: (i) integrate the expression cassette into a less active region of the genome (Galizi *et al.*, 2014); (ii) introduce mutations to the promoter region, such as the addition of spacer between TFBSs (Simoni *et al.*, 2020); (iii) introduce primary ORFs to substantially deduce translation efficiency (Southall *et al.*, 2013); (iv) introduce PEST sequences or other protein destabilization domains (Sethi and Wang, 2017; Kogenaru and Isalan, 2018); or (v) identify a novel promoter with a similar but dampened expression profile using expression datasets (see section 3.9, 'Mining Transcriptomics Data for Promoter Design', above).

3.9.5 Signal peptides for subcellular and extracellular localization

Endogenous proteins typically carry signal peptides that mediate trafficking of the mature protein from its site of production in the cytosol to its final destination within or outside the cell. For this reason, experiments designed to overexpress or mis-express an endogenous gene are possible using novel promoters and enhancers without the need for modifications to the protein itself. However, non-endogenous transgenes may require the addition of N- or C-terminal signal peptides to mediate their subcellular localization, especially when using transgenes derived from viruses or prokaryotes such as Cas9, phiC31 integrase and recombinases, all of which require the addition of non-native NLS sequences to ensure their movement into the nucleus.

More complex requirements for subcellular or extracellular specificity may include subcellular localization in neurons; unlike other cell types, neurons may carry long axon and dendrite projections that lack a substantial cytoplasm in which transgenes can be expressed and visualized. This is particularly problematic for studies aiming to delineate the architecture of neurons and their projections into the brain, because expressed fluorescent proteins will be primarily limited to the cell body. This can be remedied by including signal peptides, such as from the mouse mCD4 or mCD8 genes, that are sufficient to anchor fluorescent proteins to the cell membrane in *Drosophila* and mosquitoes (Lee and Luo, 1999; Matthews *et al.*, 2019). Similarly, protein expression can be limited to the synapse to facilitate functional imaging and neuroanatomical reconstruction of the brain by including signal peptides from endogenous synaptic proteins such as *Synaptobrevin* (nSyb), *Synaptotagmin1* (Syt1), *bruchpilot* (brp) and *embryonic lethal abnormal vision* (elav) (Zhao *et al.*, 2021). Unfortunately, there currently exist few peptides known to induce specific localization within cells, and experimenters may need to identify signal peptides on endogenous proteins that show the desired localization, or target these proteins using an in-frame fusion approach (see below).

3.9.6 Controlling for position effects

The degree to which canonical expression is perturbed by a position effect can vary greatly, from sex specificity conferred by integration within a sex chromosome to low or undetectable levels of expression by integration within a heterochromatic region of the genome.

To mitigate against position effects, experimenters often generate several independent strains integrated at different genomic loci by using transposases, such

that the profile of transgene expression can be compared between strains. Alternatively, transgenes can be inserted at specific loci using CRISPR-mediated HDR (see Ahmed and Wimmer, Chapter 5, this volume) (Gantz *et al*., 2015; Kistler *et al*., 2015; Hammond *et al*., 2016) or phiC31 integrase (see Ahmed and Wimmer, Chapter 5, this volume) (Nimmo *et al*., 2006; Meredith *et al*., 2011; Haghighat-Khah *et al*., 2015; Hammond *et al*., 2016), the latter being used to insert transgenes into a 'docking' site previously modified to contain one or two attP sites. Defined loci, such as docking sites, are usually chosen for their lack of position effects (Volohonsky *et al*., 2015), but some may impart robust and well-characterized modifications that can be used to generate desirable and potentially unique patterns of transgene expression, such as male specificity conferred by integration onto the *Anopheles* Y-chromosome (Bernardini *et al*., 2014).

Insulator sequences, and the insulator proteins they recruit, may further limit the action of nearby enhancers, silencers and heterochromatin on gene expression through the formation of DNA loops or nucleosome modifications. Five core insulator proteins have been described in *D. melanogaster* and several have known orthologues in mosquitoes, including Su(Hw), dCTCF, CP190 and GAF (Gray and Coates, 2005; Carballar-Lejarazu *et al*., 2013). The best described insulator is the 388 bp *gypsy* element isolated from the *D. melanogaster* gypsy retrotransposon which, like other insulators, relies upon binding of its core insulator protein Su(Hw) and supporting complex proteins to function. Orthologues of Su(Hw) and several *gypsy* complex genes, *mod(mdg4)2.2* and *CP190*, have been identified in the genomes of four mosquito species (Carballar-Lejarazu *et al*., 2016), lending credence to the possibility that mosquito *gypsy* elements could be used to mitigate position effects. Indeed, the *gypsy* insulator element from *Drosophila* is functional in mosquitoes (Lynd and Lycett, 2012; Carballar-Lejarazu *et al*., 2013; Adolfi *et al*., 2018; Pham *et al*., 2019) and can boost expression by as much as 60-fold (Carballar-Lejarazu *et al*., 2013). Gypsy elements can protect not only against genomic position effects, but also against perturbation by other enhancers and promoters contained within the transformation construct, such as those of the marker cassette that might affect expression of an effector transgene. Whilst insulators can dampen these effects, they rarely absolve them completely and are also susceptible to certain position effects (Markstein *et al*., 2008). As such, a prudent strategy to ensure robust transgene expression may include the use of *gypsy* elements and multiple integrations into the genome, followed by visual inspection of expression (if using a marker) or transcriptomics.

3.9.7 In-frame fusions to capture endogenous regulation

To mitigate against unpredictable position effects and incomplete promoters/enhancers, several research groups have begun to use CRISPR-based HDR to target insertion of a transgene into the genome as in-frame fusions to an endogenous protein. In this way, transgene expression may fully recapitulate that of the endogenous gene and can be designed to have minimal interference of the target gene, or to induce a knockout mutation.

Depending on the intended outcome, transgenes can be tethered to the endogenous protein such that subcellular and extracellular protein trafficking can be mediated by endogenous signal peptides, although such approaches risk interfering with protein function. In-frame fusions of this type must target the C- or N-terminus of an endogenous protein such that the two are in tandem, in frame, and separated by a peptide linker (such as a GSG linker), allowing transgene localization to mimic that of the endogenous target gene. This has been especially powerful for expression and subcellular localization in neurons, where position effects commonly cause low and variegated expression for promoter-based strategies (Matthews *et al*., 2019). Alternatively, one or more proteins can be inserted as in-frame fusions that are separated by a

cleavage peptide (commonly T2A or F2A) to prevent interference of either protein with each other. This strategy has been effective for expression of haemolymph-secreted anti-parasitic effector proteins in the midgut of *An. gambiae* by targeting *carboxypeptidase A*, *adult peritrophic matrix protein 1* and *alkaline phosphatase 2* (Hoermann *et al*., 2021).

The approach has proved powerful in the study of mosquito neurobiology. Expression of GCaMP in subsets of *Ae. aegypti* neurons has been achieved by targeting in-frame fusions of *ppk301* (Matthews *et al*., 2019), *fruitless* (Basrur *et al*., 2020) and *synaptotagmin1* (*Syt1*) (Zhao *et al*., 2021). Because synaptotagmin is a synaptic protein, the C-terminal in-frame fusion also localized GCAMP to the neuronal synapse to facilitate calcium imagining and neuroanatomical reconstruction of the brain. Whilst effective at capturing highly specific subcellular specificity, protein levels may be insufficient when targeting genes with naturally low expression, such as olfactory receptors, and can be boosted by using a binary expression system.

3.9.8 Binary expression systems

Binary expression systems have recently been adapted for a broad range of non-model insects to manipulate transgene expression through the interaction of 'driver' and 'responder' elements (see Schetelig *et al*., Chapter 2, this volume). Briefly, a driver is a *trans*-activator under transcriptional control of any promoter, and whose binding to its cognate activation sequence drives expression of a downstream reporter transgene – the 'responder'. This allows a promoter to be physically separated from the reporter transgene in the genome, such that libraries of different driver and responder strains can be combined in novel combinations.

The first and most widely used binary expression system is based on the Gal4 transactivator and its upstream activation sequence (UAS), active in *D. melanogaster* (Brand and Perrimon, 1993), *Tribolium casteneum* (Schinko *et al*., 2010), *Bombyx mori* (Uchino *et al*., 2008), *An. gambiae* (Lynd and

Lycett, 2012) and *Ae. aegypti* (Kokoza and Raikhel, 2011), among others. Unfortunately, high Gal4 expression can be toxic, due to global non-specific activation of other genes, and is no longer used in *Ae. aegypti* for this reason. The recently adapted Q-system for binary expression appears to show reduced toxicity in mosquitoes, is inducible by quinic acid and benefits from a repressor that can function independent of temperature (Potter *et al*., 2010; Riabinina *et al*., 2015, Riabinina *et al*., 2016; Matthews *et al*., 2019). Both Gal4-UAS and Q-systems are available in a split format under which DNA-binding and activation domains of the transactivator can be placed under control of separate promoters (Luan *et al*., 2006; Riabinina *et al*., 2019). This allows for inter-sectional reporter expression in a highly specific and potentially novel subset of cells. These systems also allow the gain on expression to be 'toggled' by expanding or contracting the number of activation sequences in a responder, which is particularly attractive for neurobiology research where endogenous neuronal promoters tend to drive weak transgene expression.

3.10 Future Prospects

Advances in genome engineering and binary expression systems have made a dramatic impact on the potential to replicate endogenous gene expression, but also to generate novel patterns of expression.

The characterized promoters discussed here can drive expression in key tissues across insect species, but few show cell-type specificity that will be essential to dissect the molecular genetics of neurons, immune cells, germline development and embryogenesis. Single-cell RNA sequencing (scRNA-seq) will be important in characterizing such promoters and has already revealed the micro-scale transcriptional changes that take place during spermatogenesis (Taxiarchi *et al*., 2019). Similarly, single-nucleus RNA sequencing (snRNA-seq) methods have shed light on the diversity of cell populations on the mosquito midgut (Cui and

Franz, 2020). Further advances in snRNA-seq will be needed for tissues that are difficult to dissociate, such as the peripheral sensory appendages.

Computational approaches to identify and characterize promoter and enhancer sequences are also under way (Schember and Halfon, 2021) and will complement applied methods such as promoter trapping (Reid *et al.*, 2018) and enhancer trapping (O'Brochta *et al.*, 2012) that have proved useful in generating large collections of driver lines in *D. melanogaster* (Bellen *et al.*, 1989), *Bombyx mori* (Tsubota *et al.*, 2014) and *Tribolium castaneum* (Trauner *et al.*, 2009).

Novel expression patterns will also be important to manipulate subsets of cells and tissues and may be possible by expanding split-binary systems to non-model insects. Exciting new sensor technologies may achieve similar results, such as riboswitches that transition from an inactive to active state upon interaction with RNAs (Galizi *et al.*, 2020). These interactions are sequence-specific and can be programmed to respond to endogenous or exogenous RNAs, such as those derived from a pathogen. In this way, riboswitches can act as sensors. For example, a transgene placed under transcriptional control of a midgut promoter could be additionally regulated by a riboswitch programmed to respond to arbovirus RNA. Alternatively, riboswitches could be used for intersectional expression in subsets of cells.

References

Abraham, E.G., Donnelly-Doman, M., Fujioka, H., Ghosh, A., Moreira, L. and Jacobs-Lorena, M. (2005) Driving midgut-specific expression and secretion of a foreign protein in transgenic mosquitoes with AgAper1 regulatory elements. *Insect Molecular Biology* 14, 271–279.

Adelman, Z.N., Jasinskiene, N., Onal, S., Juhn, J., Ashikyan, A. *et al.* (2007) Nanos gene control DNA mediates developmentally regulated transposition in the yellow fever mosquito *Aedes aegypti*. *Proceedings of the National Academy of Sciences USA* 104(24), 9970–9975.

Adolfi, A., Gantz, V.M., Jasinskiene, N., Lee, H.F., Hwang, K., Terradas, G., Bulger, E.A., Ramaiah, A., Bennett, J.B., Emerson, J.J., Marshall, J.M., Bier, E. and James, A.A. (2020) Efficient population modification gene-drive rescue system in the malaria mosquito *Anopheles stephensi*. *Nature Communications* 11, 5553.

Adolfi, A., Pondeville, E., Lynd, A., Bourgouin, C. and Lycett, G.J. (2018) Multi-tissue GAL4-mediated gene expression in all *Anopheles gambiae* life stages using an endogenous polyubiquitin promoter. *Insect Biochemistry and Molecular Biology* 96, 1–9.

Akbari, O.S., Antoshechkin, I., Amrhein, H., Williams, B., Diloreto, R., Sandler, J. and Hay, B.A. (2013) The developmental transcriptome of the mosquito *Aedes aegypti*, an invasive species and major arbovirus vector. *G3 (Bethesda)* 3, 1493–1509.

Akbari, O.S., Papathanos, P.A., Sandler, J.E., Kennedy, K. and Hay, B.A. (2014) Identification of germline transcriptional regulatory elements in *Aedes aegypti*. *Scientific Reports* 4, 3954.

Allen, M.L. and Christensen, B.M. (2004) Flight muscle-specific expression of act88F: GFP in transgenic *Culex quinquefasciatus* Say (Diptera: Culicidae). *Parasitology International* 53(4), 307–314.

Allen, M.L., O'Brochta, D.A., Atkinson, P.W. and Levesque, C.S. (2001) Stable, germ-line transformation of *Culex quinquefasciatus* (Diptera: Culicidae). *Journal of Medical Entomology* 38(5), 701–710.

Anderson, M.A., Gross, T.L., Myles, K.M. and Adelman, Z.N. (2010) Validation of novel promoter sequences derived from two endogenous ubiquitin genes in transgenic *Aedes aegypti*. *Insect Molecular Biology* 19(4), 441–449.

Anderson, M.A.E., Purcell, J., Veerkuijl, S.A.N., Norman, V.C., Leftwich, P.T. *et al.* (2020) Expanding the CRISPR toolbox in culicine mosquitoes: in vitro validation of Pol III promoters. *ACS Synthetic Biology* 9(3), 678–681.

Anglero-Rodriguez, Y.I., Macleod, H.J., Kang, S., Carlson, J.S., Jupatanakul, N. and Dimopoulos, G. (2017) *Aedes aegypti* molecular responses to Zika virus: modulation of infection by the Toll and Jak/Stat immune pathways and virus host factors. *Frontiers in Microbiology* 8, 2050.

Aryan, A., Anderson, M.A.E., Biedler, J.K., Qi, Y., Overcash, J.M., Naumenko, A.N. *et al.* (2020) Nix alone is sufficient to convert female *Aedes aegypti* into fertile males and myo-sex is needed for male flight. *Proceedings of the National Academy of Sciences USA* 117, 17702–17709.

Baker, D.A., Nolan, T., Fischer, B., Pinder, A., Crisanti, A. and Russell, S. (2011) A comprehensive gene expression atlas of sex- and tissue-specificity in the malaria vector, *Anopheles gambiae*. *BMC Genomics* 12, 296.

Basrur, N.S., De Obaldia, M.E., Morita, T., Herre, M., Von Heynitz, R.K. *et al.* (2020) Fruitless mutant male mosquitoes gain attraction to human odor. *Elife,* 9.

Behura, S.K. and Severson, D.W. (2011) Coadaptation of isoacceptor tRNA genes and codon usage bias for translation efficiency in *Aedes aegypti* and *Anopheles gambiae*. *Insect Molecular Biology* 20**,** 177–187.

Behura, S.K. and Severson, D.W. (2012) Comparative analysis of codon usage bias and codon context patterns between dipteran and hymenopteran sequenced genomes. *PLoS One* 7, e43111.

Behura, S.K., Gomez-Machorro, C., Harker, B.W., Debruyn, B., Lovin, D.D. *et al.* (2011) Global cross-talk of genes of the mosquito *Aedes aegypti* in response to dengue virus infection. *PLoS Neglected Tropical Diseases* 5, e1385.

Behura, S.K., Sarro, J., Li, P., Mysore, K., Severson, D.W. *et al.* (2016) High-throughput *cis*-regulatory element discovery in the vector mosquito *Aedes aegypti*. *BMC Genomics* 17, 341.

Bellen, H.J., O'Kane, C.J., Wilson, C., Grossniklaus, U., Pearson, R.K. and Gehring, W.J. (1989) P-element-mediated enhancer detection: a versatile method to study development in *Drosophila*. *Genes & Development* 3, 1288–1300.

Bernardini, F., Galizi, R., Menichelli, M., Papathanos, P.A., Dritsou, V. *et al.* (2014) Site-specific genetic engineering of the *Anopheles gambiae* Y chromosome. *Proceedings of the National Academy of Sciences USA* 111, 7600–7605.

Bernardini, F., Haghighat-Khah, R.E., Galizi, R., Hammond, A.M., Nolan, T. and Crisanti, A. (2018) Molecular tools and genetic markers for the generation of transgenic sexing strains in Anopheline mosquitoes. *Parasites & Vectors* 11, 660.

Betting, V., Joosten, J., Halbach, R., Thaler, M., Miesen, P. and Van Rij, R.P. (2021) A piRNA-lncRNA regulatory network initiates responder and trailer piRNA formation during mosquito embryonic development. *RNA Journal* [published in advance, 1 July 2021]. doi: 10.1261/rna.078876.121

Biedler, J.K., Hu, W., Tae, H. and Tu, Z. (2012) Identification of early zygotic genes in the yellow fever mosquito *Aedes aegypti* and discovery of a motif involved in early zygotic genome activation. *PLoS ONE* 7, e33933.

Bonizzoni, M., Dunn, W.A., Campbell, C.L., Olson, K.E., Dimon, M.T. *et al.* (2011) RNA-seq analyses of blood-induced changes in gene expression in the mosquito vector species, *Aedes aegypti. BMC Genomics* 12, 82.

Brand, A.H. and Perrimon, N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118, 401–415.

Brennecke, J., Aravin, A.A., Stark, A., Dus, M., Kellis, M. *et al.* (2007) Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. *Cell* 128, 1089–1103.

Brown, J.B., Boley, N., Eisman, R., May, G.E., Stoiber, M.H. *et al.* (2014) Diversity and dynamics of the *Drosophila* transcriptome. *Nature* 512, 393–399.

Bryant, W.B., Mills, M.K., Olson, B.J. and Michel, K. (2019) Small RNA-seq analysis reveals miRNA expression dynamics across tissues in the malaria vector, *Anopheles gambiae*. *G3: Genes, Genomes, Genetics* 9**,** 1507–1517.

Bryant, W.B., Ray, S. and Mills, M.K. (2020) Global analysis of small non-coding RNA populations across tissues in the malaria vector, *Anopheles gambiae. Insects* 11(7), 406.

Caragata, E.P., Poinsignon, A., Moreira, L.A., Johnson, P.H., Leong, Y.S. *et al.* (2011) Improved accuracy of the transcriptional profiling method of age grading in *Aedes aegypti* mosquitoes under laboratory and semi-field cage conditions and in the presence of Wolbachia infection. *Insect Molecular Biology* 20, 215–224.

Carballar-Lejarazu, R., Jasinskiene, N. and James, A.A. (2013) Exogenous gypsy insulator sequences modulate transgene expression in the malaria vector mosquito, *Anopheles stephensi*. *Proceedings of the National Academy of Sciences USA* 110, 7176–7181.

Carballar-Lejarazu, R., Brennock, P. and James, A.A. (2016) Suppressor of hairy-wing, modifier of mdg4 and centrosomal protein of 190 gene orthologues of the gypsy insulator complex in the malaria mosquito, *Anopheles stephensi*. *Insect Molecular Biology* 25, 460–469.

Carballar-Lejarazu, R., Ogaugwu, C., Tushar, T., Kelsey, A., Pham, T. B. *et al.* (2020) Next-generation gene drive for population modification of the malaria vector mosquito, *Anopheles gambiae*. *Proceedings of the National Academy of Sciences USA* 117, 22805–22814.

Cassone, B.J., Kay, R.G., Daugherty, M.P. and White, B.J. (2017) Comparative transcriptomics of malaria mosquito testes: function, evolution, and linkage. *G3: Genes, Genomes, Genetics* 7, 1127–1136.

Catteruccia, F., Nolan, T., Loukeris, T.G., Blass, C., Savakis, F.C. *et al.* (2000) Stable germline transformation of the malaria mosquito *Anopheles stephensi*. *Nature* 405(6789), 9959–962.

Catteruccia, F., Benton, J.P. and Crisanti, A. (2005) An *Anopheles* transgenic sexing strain for vector control. *Nature Biotechnology* 23, 1414–1417.

Chen, X.G., Marinotti, O., Whitman, L., Jasinskiene, N., James, A.A. and Romans, P. (2007) The *Anopheles gambiae* vitellogenin gene (VGT2) promoter directs persistent accumulation of a reporter gene product in transgenic *Anopheles stephensi* following multiple bloodmeals. *American Journal of Tropical Medicine and Hygiene* 76, 1118–1124.

Cho, K.H., Cheon, H.M., Kokoza, V. and Raikhel, A.S. (2006) Regulatory region of the vitellogenin receptor gene sufficient for high-level, germ line cell-specific ovarian expression in transgenic *Aedes aegypti* mosquitoes. *Insect Biochemistry and Molecular Biology* 36(4), 273–281.

Choi, Y.J., Fuchs, J.F., Mayhew, G.F., Yu, H.E. and Christensen, B.M. (2012) Tissue-enriched expression profiles in *Aedes aegypti* identify hemocyte-specific transcriptome responses to infection. *Insect Biochemistry and Molecular Biology* 42, 729–738.

Coates, C.J., Jasinskiene, N., Pott, G.B. and James, A.A. (1999). Promoter-directed expression of recombinant fire-fly luciferase in the salivary glands of Hermes-transformed *Aedes aegypti*. *Gene* 226, 317–325.

Cook, P.E. and Sinkins, S.P. (2010) Transcriptional profiling of *Anopheles gambiae* mosquitoes for adult age estimation. *Insect Molecular Biology* 19, 745–751.

Criscione, F., Qi, Y., Saunders, R., Hall, B. and Tu, Z. (2013) A unique Y gene in the Asian malaria mosquito *Anopheles stephensi* encodes a small lysine-rich protein and is transcribed at the onset of embryonic development. *Insect Molecular Biology* 22, 433–441.

Cui, Y. and Franz, A.W.E. (2020) Heterogeneity of midgut cells and their differential responses to blood meal ingestion by the mosquito, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* 127, 103496.

Danino, Y.M., Even, D., Ideses, D. and Juven-Gershon, T. (2015) The core promoter: at the heart of gene expression. *Biochimica et Biophysica Acta* 1849, 1116–1131.

Dissanayake, S.N., Ribeiro, J.M., Wang, M.H., Dunn, W.A., Yan, G., James, A.A. and Marinotti, O. (2010) aeGEPUCI: a database of gene expression in the dengue vector mosquito, *Aedes aegypti*. *BMC Research Notes* 3, 248.

Dong, S., Lin, J., Held, N.L., Clem, R.J., Passarelli, A.L. and Franz, A.W. (2015) Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, *Aedes aegypti*. *PLoS ONE* 10(3), e0122353.

Drysdale, R. and Flybase Consortium (2008) FlyBase: a database for the *Drosophila* research community. *Methods in Molecular Biology* 420, 45–59.

Etebari, K., Osei-Amo, S., Blomberg, S.P. and Asgari, S. (2015) Dengue virus infection alters post-transcriptional modification of microRNAs in the mosquito vector *Aedes aegypti*. *Scientific Reports* 5, 15968.

Faucon, F., Gaude, T., Dusfour, I., Navratil, V., Corbel, V., Juntarajumnong, W., Girod, R., Poupardin, R., Boyer, F., Reynaud, S. and David, J.P. (2017) In the hunt for genomic markers of metabolic resistance to pyrethroids in the mosquito *Aedes aegypti*: an integrated next-generation sequencing approach. *PLoS Neglected Tropical Diseases* 11, e0005526.

Feng, X., Lopez Del Amo, V., Mameli, E., Lee, M., Bishop, A.L. *et al.* (2021) Optimized CRISPR tools and site-directed transgenesis towards gene drive development in *Culex quinquefasciatus* mosquitoes. *Nature Communications* 12(1), 2960.

Fu, G., Lees, R.S., Nimmo, D., Aw, D., Jin, L. *et al.* (2010) Female-specific flightless phenotype for mosquito control. *Proceedings of the National Academy of Sciences USA* 107(1), 4550–4554.

Gabrieli, P., Kakani, E.G., Mitchell, S.N., Mameli, E., Want, E.J. *et al.* (2014) Sexual transfer of the steroid hormone 20E induces the postmating switch in *Anopheles gambiae*. *Proceedings of the National Academy of Sciences USA* 111, 16353–16358.

Galizi, R., Doyle, L.A., Menichelli, M., Bernardini, F., Deredec, A. *et al.* (2014) A synthetic sex ratio distortion system for the control of the human malaria mosquito. *Nature Communications* 5, 3977.

Galizi, R., Duncan, J.N., Rostain, W., Quinn, C.M., Storch, M. *et al.* (2020) Engineered RNA-interacting CRISPR guide RNAs for genetic sensing and diagnostics. *The CRISPR Journal* 3, 398–408.

Gallegos, J.E. and Rose, A.B. (2017) Intron DNA sequences can be more important than the proximal promoter in determining the site of transcript initiation. *Plant Cell* 29, 843–853.

Gantz, V.M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V.M. *et al.* (2015) Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. *Proceedings of the National Academy of Sciences USA* 112, E6736–6743.

Giraldo-Calderon, G.I., Emrich, S.J., Maccallum, R.M., Maslen, G., Dialynas, E. *et al.* (2015) VectorBase: an updated bioinformatics resource for invertebrate vectors and other organisms related with human diseases. *Nucleic Acids Research* 43, D707–713.

Goltsev, Y., Rezende, G.L., Vranizan, K., Lanzaro, G., Valle, D. and Levine, M. (2009) Developmental and evolutionary basis for drought tolerance of the *Anopheles gambiae* embryo. *Developmental Biology* 330, 462–470.

Gomez-Diaz, E., Rivero, A., Chandre, F. and Corces, V.G. (2014) Insights into the epigenomic landscape of the human malaria vector *Anopheles gambiae*. *Frontiers in Genetics* 5, 277.

Gray, C.E. and Coates, C.J. (2005) Cloning and characterization of cDNAs encoding putative CTCFs in the mosquitoes, *Aedes aegypti* and *Anopheles gambiae*. *BMC Molecular Biology* 6, 16.

Groth, A.C., Fish, M., Nusse, R. and Caolos, M.P. (2004) Construction of transgenic *Drosophila* by using the site-specific integrase from phage phiC31. *Genetics* 166(4), 1775–1782.

Haghighat-Khah, R.E., Scaife, S., Martins, S., St John, O., Matzen, K.J. *et al.* (2015) Site-specific cassette exchange systems in the *Aedes aegypti* mosquito and the *Plutella xylostella* moth. *PLoS ONE* 10, e0121097.

Hall, A.B., Basu, S., Jiang, X., Qi, Y., Timoshevskiy, V.A. *et al.* (2015) SEX DETERMINATION. A male-determining factor in the mosquito *ano*. *Science* 348, 1268–1270.

Hammond, A.M. and Galizi, R. (2017) Gene drives to fight malaria: current state and future directions. *Pathogens and Global Health* 111, 412–423.

Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C. *et al.* (2016) A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nature Biotechnology* 34, 78–83.

Hammond, A., Karlsson, X., Morianou, I., Kyrou, K., Beaghton, A. *et al.* (2021) Regulating the expression of gene drives is key to increasing their invasive potential and the mitigation of resistance. *PLoS Genetics* 17, e1009321.

Harker, B.W., Behura, S.K., Debruyn, B.S., Lovin, D.D., Mori, A. *et al.* (2013) Stage-specific transcription during development of *Aedes aegypti*. *BMC Developmental Biology* 13, 29.

Hodges, T.K., Cosme, L.V., Athrey, G., Pathikonda, S., Takken, W. and Slotman, M.A. (2014) Species-specific chemosensory gene expression in the olfactory organs of the malaria vector *Anopheles gambiae*. *BMC Genomics* 15, 1089.

Hoermann, A., Tapanelli, S., Capriotti, P., Del Corsano, G., Masters, E.K. *et al.* (2021) Converting endogenous genes of the malaria mosquito into simple non-autonomous gene drives for population replacement. *Elife,* 10.

Horn, C. Jaunich, B. and Wimmer, E.A. (2000) Highly sensitive, fluorescent transformation marker for *Drosophila* transgenesis. *Development Genes and Evolution* 210(12), 630–637. doi: 10.1007/s004270000111

Hu, W. and Tu, Z.J. (2018) Functional analysis of the promoter of an early zygotic gene KLC2 in *Aedes aegypti*. *Parasites & Vectors* 11, 655.

Isaacs, A.T., Jasinskiene, N., Tretiakov, M., Thiery, I., Zettor, A. *et al.* (2012) Transgenic *Anopheles stephensi* coexpressing single-chain antibodies resist *Plasmodium falciparum* development. *Proceedings of the National Academy of Sciences USA* 109, E1922–1930.

Isaacs, A.T., Mawejje, H.D., Tomlinson, S., Rigden, D.J. and Donnelly, M.J. (2018) Genome-wide transcriptional analyses in *Anopheles* mosquitoes reveal an unexpected association between salivary gland gene expression and insecticide resistance. *BMC Genomics* 19, 225.

Isoe, J., Kunz, S., Manhart, C., Wells, M.A. and Miesfeld, R.L. (2007) Regulated expression of microinjected DNA in adult *Aedes aegypti* mosquitoes. *Insect Molecular Biology* 16, 83–92.

Ito, J., Ghosh, A., Moreira, L.A., Wimmer, E.A. and Jacobs-Lorena, M. (2002) Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature* 417, 452–455.

Izquierdo, A., Fahrenberger, M., Persampieri, T., Benedict, M.Q., Giles, T. *et al.* (2019) Evolution of gene expression levels in the male reproductive organs of *Anopheles* mosquitoes. *Life Science Alliance* 2, e201800191.

Jasinskiene, N., Coleman, J., Ashikyan, A., Salampessy, M., Marinotti, O. and James, A.A. (2007) Genetic control of malaria parasite transmission: threshold levels for infection in an avian model system. *American Journal of Tropical Medicine and Hygiene* 76(6), 1072–1078.

Jenkins, A.M., Waterhouse, R.M. and Muskavitch, M.A. (2015) Long non-coding RNA discovery across the genus *Anopheles* reveals conserved secondary structures within and beyond the Gambiae complex. *BMC Genomics* 16, 337.

Jiang, X., Biedler, J.K., Qi, Y., Hall, A.B. and Tu, Z. (2015) Complete dosage compensation in *Anopheles stephensi* and the evolution of sex-biased genes in mosquitoes. *Genome Biology and Evolution* 7, 1914–1924.

Jin, L., Walker, A.S., Fu, G., Harvey-Samuel, T., Dafa'alla, T. *et al.* (2013) Engineered female-specific lethality for control of pest Lepidoptera. *ACS Synthetic Biology* 2, 160–166.

Kambris, Z., Cook, P.E., Phuc, H.K. and Sinkins, S.P. (2009) Immune activation by life-shortening Wolbachia and reduced filarial competence in mosquitoes. *Science* 326, 134–136.

Kandul, N.P., Liu, J., Bennett, J.B., Marshall, J.M. and Akbari, O.S. (2021) A confinable home-and-rescue gene drive for population modification. *Elife* 10.

Kim, W., Koo, H., Richman, A.M., Seeley, D., Vizioli, J. *et al.* (2004) Ectopic expression of a cecropin transgene in the human malaria vector mosquito *Anopheles gambiae* (Diptera: Culicidae): effects on susceptibility to Plasmodium. *Journal of Medical Entomology* 41, 447–455.

Kistler, K.E., Vosshall, L.B. and Matthews, B.J. (2015) Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*. *Cell Reports* 11, 51–60.

Kogenaru, M. and Isalan, M. (2018) Drug-inducible control of lethality genes: a low background destabilizing domain architecture applied to the Gal4-UAS system in *Drosophila*. *ACS Synthetic Biology* 7, 1496–1506.

Kojin, B.B., Biedler, J.K., Tu, Z. and Adelman, Z.N. (2020) Characterization of a female germline and early zygote promoter from the transcription factor bZip1 in the dengue mosquito *Aedes aegypti*. *Parasites & Vectors* 13, 353.

Kokoza, V.A. and Raikhel, A.S. (2011) Targeted gene expression in the transgenic *Aedes aegypti* using the binary Gal4-UAS system. *Insect Biochemistry and Molecular Biology* 41(8), 637–644.

Kokoza, V., Ahmed, A., Wimmer, E.A. and Raikhel, A.S. (2001a) Efficient transformation of the yellow fever mosquito *Aedes aegypti* using the piggyBac transposable element vector pBac[3xP3-EGFP afm]. *Insect Biochemistry and Molecular Biology* 31(12), 1137–1143.

Kokoza, V.A., Martin, D., Mienaltowski, M.J., Ahmed, A., Morton, C.M. and Raikhel, A.S. (2001b) Transcriptional regulation of the mosquito vitellogenin gene via a blood meal-triggered cascade. *Gene* 274, 47–65.

Komitopoulou, K., Christophides, G.K., Kalosaka, K., Chrysanthis, G., Theodoraki, M.A. *et al.* (2004) Medfly promoters relevant to the sterile insect technique. *Insect Biochemistry and Molecular Biology* 34, 149–157.

Konet, D.S., Anderson, J., Piper, J., Akkina, R., Suchman, E. and Carlson, J. (2007) Short-hairpin RNA expressed from polymerase III promoters mediates RNA interference in mosquito cells. *Insect Molecular Biology* 16(2), 199–206.

Koutsos, A.C., Blass, C., Meister, S., Schmidt, S., Maccallum, R.M. *et al.* (2007) Life cycle transcriptome of the malaria mosquito *Anopheles gambiae* and comparison with the fruitfly *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences USA* 104, 11304–11309.

Labbé, G.M., Nimmo, D.D. and Alphey, L. (2010) piggybac and PhiC31-mediated genetic transformation of the Asian tiger mosquito, *Aedes albopictus* (Skuse). *PLoS Neglected Tropical Diseases* 4(8), e788.

Labbé, G.M., Scaife, S., Morgan, S.A., Curtis, Z.H. and Alphey, L. (2012) Female-specific flightless (fsRIDL) phenotype for control of *Aedes albopictus*. *PLoS Neglected Tropical Diseases* 6(7), E1724.

Leader, D.P., Krause, S.A., Pandit, A., Davies, S.A. and Dow, J.A.T. (2018) FlyAtlas 2: a new version of the *Drosophila melanogaster* expression atlas with RNA-Seq, miRNA-Seq and sex-specific data. *Nucleic Acids Research* 46, D809–D815.

Lee, T. and Luo, L. (1999) Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* 22, 451–461.

Leming, M.T., Rund, S.S., Behura, S.K., Duffield, G.E. and O'Tousa, J.E. (2014) A database of circadian and diel rhythmic gene expression in the yellow fever mosquito *Aedes aegypti*. *BMC Genomics* 15, 1128.

Lezcano, O.M., Sanchez-Polo, M., Ruiz, J.L. and Gomez-Diaz, E. (2020) Chromatin structure and function in mosquitoes. *Frontiers in Genetics* 11, 602949.

Li, M., Yang, T., Kandul, N.P., Bui, M., Gamez, S. *et al.* (2020) Development of a confinable gene drive system in the human disease vector *Aedes aegypti*. *Elife,* 9.

Li, Y., Piermarini, P.M., Esquivel, C.J., Price, D.P., Drumm, H.E. *et al.* (2017) RNA-Seq comparison of larval and adult malpighian tubules of the yellow fever mosquito *Aedes aegypti* reveals life stage-specific changes in renal function. *Frontiers in Physiology* 8, 283.

Lombardo, F., Di Cristina, M., Spanos, L., Louis, C., Coluzzi, M. and Arca, B. (2000) Promoter sequences of the putative *Anopheles gambiae* apyrase confer salivary gland expression in *Drosophila melanogaster*. *Journal of Biological Chemistry* 275, 23861–23868.

Lombardo, F., Nolan, T., Lycett, G., Lanfrancotti, A., Stich, N. *et al.* (2005) An *Anopheles gambiae* salivary gland promoter analysis in *Drosophila melanogaster* and *Anopheles stephensi*. *Insect Molecular Biology* 14, 207–216.

Lombardo, F., Lycett, G.J., Lanfrancotti, A., Coluzzi, M. and Arca, B. (2009) Analysis of apyrase 5' upstream region validates improved *Anopheles gambiae* transformation technique. *BMC Research Notes* 2, 24.

Lombardo, F., Ghani, Y., Kafatos, F.C. and Christophides, G.K. (2013) Comprehensive genetic dissection of the hemocyte immune response in the malaria mosquito *Anopheles gambiae*. *PLoS Pathogens* 9, e1003145.

Lott, S.E., Villalta, J.E., Schroth, G.P., Luo, S., Tonkin, L.A. and Eisen, M.B. (2011) Noncanonical compensation of zygotic X transcription in early *Drosophila melanogaster* development revealed through single-embryo RNA-seq. *PLoS Biology* 9, e1000590.

Luan, H., Peabody, N.C., Vinson, C. R. and White, B. H. (2006) Refined spatial manipulation of neuronal function by combinatorial restriction of transgene expression. *Neuron* 52, 425–436.

Lynd, A. and Lycett, G.J. (2012) Development of the bi-partite Gal4-UAS system in the African malaria mosquito, *Anopheles gambiae*. *PLoS ONE* 7, e31552.

Ma, Q., Srivastav, S.P., Gamez, S., Dayama, G., Feitosa-Suntheimer, F. *et al.* (2021) A mosquito small RNA genomics resource reveals dynamic evolution and host responses to viruses and transposons. *Genome Research* 31, 512–528.

Macias, V.M., Jimenez, A.J., Burini-Kojin, B., Pledger, N., Jasinskiene, N. *et al.* (2017) nanos-Driven expression of piggyBac transposase induces mobilization of a synthetic autonomous transposon in the malaria vector mosquito, *Anopheles stephensi*. *Insect Biochemistry and Molecular Biology* 87, 81–89.

Magnusson, K., Mendes, A.M., Windbichler, N., Papathanos, P.A., Nolan, T. *et al.* (2011) Transcription regulation of sex-biased genes during ontogeny in the malaria vector *Anopheles gambiae*. *PLoS ONE* 6, e21572.

Maïga, H., Damiens, D., Niang, A., Sawadogo, S.P., Fatherhaman, O. *et al.* (2014) Mating competitiveness of sterile male *Anopheles coluzzii* in large cages. *Malaria Journal,* 460.

Marinotti, O., Nguyen, Q.K., Calvo, E., James, A.A. and Ribeiro, J.M. (2005) Microarray analysis of genes showing variable expression following a blood meal in *Anopheles gambiae*. *Insect Molecular Biology* 14, 365–373.

Marinotti, O., Jasinskiene, N., Fazekas, A., Scaife, S., Fu, G. *et al.* (2013) Development of a population suppression strain of the human malaria vector mosquito, *Anopheles stephensi*. *Malaria Journal* 12, 142.

Markstein, M., Pitsouli, C., Villalta, C., Celniker, S.E. and Perrimon, N. (2008) Exploiting position effects and the gypsy retrovirus insulator to engineer precisely expressed transgenes. *Nature Genetics* 40, 476–483.

Matthews, B.J., Mcbride, C.S., Degennaro, M., Despo, O. and Vosshall, L.B. (2016) The neurotranscriptome of the *Aedes aegypti* mosquito. *BMC Genomics* 17, 32.

Matthews, B.J., Younger, M.A. and Vosshall, L.B. (2019) The ion channel ppk301 controls freshwater egg-laying in the mosquito *Aedes aegypti*. *Elife,* 8.

McBride, C.S., Baier, F., Omondi, A.B., Spitzer, S.A., Lutomiah, J. *et al.* (2014) Evolution of mosquito preference for humans linked to an odorant receptor. *Nature* 515, 222–227.

Meccariello, A., Salvemini, M., Primo, P., Hall, B., Koskinioti, P. *et al.* (2019) Maleness-on-the-Y (MoY) orchestrates male sex determination in major agricultural fruit fly pests. *Science* 365, 1457–1460.

Meccariello, A., Krsticevic, F., Colonna, R., Del Corsano, G., Fasulo, B. *et al.* (2021) Engineered sex ratio distortion by X-shredding in the global agricultural pest *Ceratitis capitata*. *BMC Biology* 19(1), 78.

Mendes, A.M., Awono-Ambene, P.H., Nsango, S.E., Cohuet, A., Fontenille, D. *et al.* (2011) Infection intensity-dependent responses of *Anopheles gambiae* to the African malaria parasite *Plasmodium falciparum*. *Infection and Immunity* 79, 4708–4715.

Meredith, J.M., Basu, S., Nimmo, D.D., Larget-Thiery, I., Warr, E.L. *et al.* (2011) Site-specific integration and expression of an anti-malarial gene in transgenic *Anopheles gambiae* significantly reduces *Plasmodium* infections. *PLoS ONE* 6, e14587.

Meredith, J.M., Underhill, A., McArthur, C.C. and Eggleston, P. (2013) Next-generation site-directed transgenesis in the malaria vector mosquito *Anopheles gambiae*: self-docking strains expressing germline-specific phiC31 integrase. *PLoS ONE* 8(3), E59264.

Moreira, L.A., Edwards, M.J., Adhami, F., Jasinskiene, N., James, A.A. and Jacobs-Lorena, M. (2000) Robust gut-specific gene expression in transgenic *Aedes aegypti* mosquitoes. *Proceedings of the National Academy of Sciences USA* 97, 10895–10898.

Mysore, K., Li, P. and Duman-Scheel, M. (2018) Identification of *Aedes aegypti* cis-regulatory elements that promote gene expression in olfactory receptor neurons of distantly related dipteran insects. *Parasites & Vectors* 11, 406.

Nardini, L., Holm, I., Pain, A., Bischoff, E., Gohl, D.M. *et al.* (2019) Influence of genetic polymorphism on transcriptional enhancer activity in the malaria vector *Anopheles* coluzzii. *Scientific Reports* 9, 15275.

Neira Oviedo, M., Vanekeris, L., Corena-Mcleod, M.D. and Linser, P.J. (2008) A microarray-based analysis of transcriptional compartmentalization in the alimentary canal of *Anopheles gambiae* (Diptera: Culicidae) larvae. *Insect Molecular Biology* 17, 61–72.

Neira-Oviedo, M., Tsyganov-Bodounov, A., Lycett, G.J., Kokoza, V., Raikhel, A.S. and Krzywinski, J. (2011) The RNA-Seq approach to studying the expression of mosquito mitochondrial genes. *Insect Molecular Biology* 20, 141–152.

Nimmo, D.D., Alphey, L., Meredith, J.M. and Eggleston, P. (2006) High efficiency site-specific genetic engineering of the mosquito genome. *Insect Molecular Biology* 15, 129–136.

Nirmala, X., Marinotti, O., Sandoval, J.M., Phin, S., Gakhar, S. *et al.* (2006) Functional characterization of the promoter of the vitellogenin gene, AsVg1, of the malaria vector, *Anopheles stephensi*. *Insect Biochemistry and Molecular Biology* 36, 694–700.

Nolan, T., Petris, E., Muller, H.M., Cronin, A., Catteruccia, F. and Crisanti, A. (2011) Analysis of two novel midgut-specific promoters driving transgene expression in *Anopheles stephensi* mosquitoes. *PLoS ONE* 6, e16471.

O'Brochta, D.A., Pilitt, K.L., Harrell, R.A. 2nd, Aluvihare, C. and Alford, R.T. (2012) Gal4-based enhancer-trapping in the malaria mosquito *Anopheles stephensi*. *G3: Genes, Genomes, Genetics* 2, 1305–1315.

Panigrahi, A. and O'Malley, B.W. (2021) Mechanisms of enhancer action: the known and the unknown. *Genome Biology* 22, 108.

Papa, F., Windbichler, N., Waterhouse, R.M., Cagnetti, A., D'Amato, R. *et al.* (2017) Rapid evolution of female-biased genes among four species of *Anopheles* malaria mosquitoes. *Genome Research* 27, 1536–1548.

Papathanos, P.A., Windbichler, N., Menichelli, M., Burt, A. and Crisanti, A. (2009) The vasa regulatory region mediates germline expression and maternal transmission of proteins in the malaria mosquito *Anopheles gambiae*: a versatile tool for genetic control strategies. *BMC Molecular Biology* 10, 65.

Pascini, T.V. and Martins, G.F. (2017) The insect spermatheca: an overview. *Zoology (Jena)* 121, 56–71.

Perez-Zamorano, B., Rosas-Madrigal, S., Lozano, O.A.M., Castillo Mendez, M. and Valverde-Garduno, V. (2017) Identification of *cis*-regulatory sequences reveals potential participation of lola and Deaf1 transcription factors in *Anopheles gambiae* innate immune response. *PLoS ONE* 12, e0186435.

Petra, O.P., Harrell, I.R. and Handler, A.M. (2002) Germ-line transformation of the South American malaria vector, *Anopheles albimanus*, with a piggyBac/EGFP transposon vector is routine and highly efficient. *Insect Molecular Biology* 11(4), 291–297.

Pfeiffer, B.D., Ngo, T.T., Hibbard, K.L., Murphy, C., Jenett, A. *et al.* (2010) Refinement of tools for targeted gene expression in *Drosophila*. *Genetics* 186, 735–755.

Pfeiffer, B.D., Truman, J.W. and Rubin, G.M. (2012) Using translational enhancers to increase transgene expression in *Drosophila*. *Proceedings of the National Academy of Sciences USA* 109, 6626–6631.

Pham, T.B., Phong, C.H., Bennett, J.B., Hwang, K., Jasinskiene, N. *et al.* (2019) Experimental population modification of the malaria vector mosquito, *Anopheles stephensi*. *PLoS Genetics* 15, e1008440.

Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J. *et al.* (2007) Late-acting dominant lethal genetic systems and mosquito control. *BMC Biology* 5, 11.

Pinkerton, A.C., Michel, K., O'Brochta, D.A. and Atkinson, P.W. (2000) Green fluorescent protein as a genetic marker in transgenic *Aedes aegypti*. *Insect Molecular Biology* 9(1), 1–10.

Pinto, S.B., Lombardo, F., Koutsos, A.C., Waterhouse, R.M., Mckay, K. *et al.* (2009) Discovery of Plasmodium modulators by genome-wide analysis of circulating hemocytes in *Anopheles gambiae*. *Proceedings of the National Academy of Sciences USA* 106, 21270–21275.

Pitts, R.J., Rinker, D.C., Jones, P.L., Rokas, A. and Zwiebel, L.J. (2011) Transcriptome profiling of chemosensory appendages in the malaria vector *Anopheles gambiae* reveals tissue- and sex-specific signatures of odor coding. *BMC Genomics* 12, 271.

Pondeville, E., Puchot, N., Parvy, J.P., Carissimo, G., Poidevin, M. *et al.* (2020) Hemocyte-targeted gene expression in the female malaria mosquito using the hemolectin promoter from *Drosophila*. *Insect Biochemistry and Molecular Biology* 120, 103339.

Potter, C.J., Tasic, B., Russler, E.V., Liang, L. and Luo, L. (2010) The Q system: a repressible binary system for transgene expression, lineage tracing, and mosaic analysis. *Cell* 141, 536–548.

Poupardin, R., Riaz, M.A., Jones, C.M., Chandor-Proust, A., Reynaud, S. and David, J.P. (2012) Do pollutants affect insecticide-driven gene selection in mosquitoes? Experimental evidence from transcriptomics. *Aquatic Toxicology* 114–115, 49–57.

Price, D.C., Egizi, A. and Fonseca, D.M. (2015) The ubiquity and ancestry of insect doublesex. *Scientific Reports* 5, 13068.

Quinn, C., Anthousi, A., Wondji, C. and Nolan, T. (2021) CRISPR-mediated knock-in of transgenes into the malaria vector *Anopheles funestus*. *G3: Genes, Genomes, Genetics* 11(8).

Rangan, P., Degennaro, M., Jaime-Bustamante, K., Coux, R.X., Martinho, R.G. and Lehmann, R. (2009) Temporal and spatial control of germ-plasm RNAs. *Current Biology* 19, 72–77.

Raquin, V., Merkling, S.H., Gausson, V., Moltini-Conclois, I., Frangeul, L. *et al.* (2017) Individual co-variation between viral RNA load and gene expression reveals novel host factors during early dengue virus infection of the *Aedes aegypti* midgut. *PLoS Neglected Tropical Diseases* 11, e0006152.

Reid, W., Pilitt, K., Alford, R., Cervantes-Medina, A., Yu, H. *et al.* (2018) An *Anopheles stephensi* promoter-trap: augmenting genome annotation and functional genomics. *G3: Genes, Genomes, Genetics* 8, 3119–3130.

Ren, X., Hoiczyk, E. and Rasgon, J.L. (2008) Viral paratransgenesis in the malaria vector *Anopheles gambiae*. *PLoS Pathogens* 4(8), e1000135.

Riabinina, O., Luginbuhl, D., Marr, E., Liu, S., Wu, M.N. *et al.* (2015) Improved and expanded Q-system reagents for genetic manipulations. *Nature Methods* 12, 219–222.

Riabinina, O., Task, D., Marr, E., Lin, C.C., Alford, R. *et al.* (2016) Organization of olfactory centres in the malaria mosquito *Anopheles gambiae*. *Nature Communications* 7, 13010.

Riabinina, O., Vernon, S.W., Dickson, B.J. and Baines, R.A. (2019) Split-QF system for fine-tuned transgene expression in *Drosophila*. *Genetics* 212**,** 53–63.

Riaz, M.A., Chandor-Proust, A., Dauphin-Villemant, C., Poupardin, R., Jones, C.M. *et al.* (2013) Molecular mechanisms associated with increased tolerance to the neonicotinoid insecticide imidacloprid in the dengue vector *Aedes aegypti*. *Aquatic Toxicology* 126, 326–337.

Ribeiro, J.M., Martin-Martin, I., Arca, B. and Calvo, E. (2016) A deep insight into the sialome of male and female *Aedes aegypti* mosquitoes. *PLoS ONE* 11, e0151400.

Rice, G.R., Barmina, O., Luecke, D., Hu, K., Arbeitman, M. and Kopp, A. (2019) Modular tissue-specific regulation of doublesex underpins sexually dimorphic development in *Drosophila*. *Development* 146.

Rinker, D.C., Zhou, X., Pitts, R.J., Consortium, A.G.C., Rokas, A. and Zwiebel, L.J. (2013) Antennal transcriptome profiles of anopheline mosquitoes reveal human host olfactory specialization in *Anopheles gambiae*. *BMC Genomics* 14, 749.

Rodrigues, F.G., Santos, M.N., De Carvalho, T.X., Rocha, B.C., Riehle, M.A. *et al.* (2008) Expression of a mutated phospholipase A2 in transgenic *Aedes fluviatilis* mosquitoes impacts *Plasmodium gallinaceum* development. *Insect Molecular Biology* 17, 175–183.

Rogers, D.W., Whitten, M.M., Thailayil, J., Soichot, J., Levashina, E.A. and Catteruccia, F. (2008) Molecular and cellular components of the mating machinery in *Anopheles gambiae* females. *Proceedings of the National Academy of Sciences USA* 105, 19390–19395.

Ruiz, J.L., Ranford-Cartwright, L.C. and Gomez-Diaz, E. (2021) The regulatory genome of the malaria vector *Anopheles gambiae*: integrating chromatin accessibility and gene expression. *NAR Genomics and Bioinformatics* 3, lqaa113.

Rund, S.S., Hou, T.Y., Ward, S.M., Collins, F.H. and Duffield, G.E. (2011) Genome-wide profiling of diel and circadian gene expression in the malaria vector *Anopheles gambiae*. *Proceedings of the National Academy of Sciences USA* 108, E421–430.

Salvemini, M., Polito, C. and Saccone, G. (2010) Fruitless alternative splicing and sex behaviour in insects: an ancient and unforgettable love story? *Journal of Genetics* 89, 287–299.

Salvemini, M., Mauro, U., Lombardo, F., Milano, A., Zazzaro, V. *et al.* A (2011) Genomic organization and splicing evolution of the doublesex gene, a *Drosophila* regulator of sexual differentiation, in the dengue and yellow fever mosquito *Aedes aegypti*. *BMC Evolutionary Biology* 11, 41.

Satyavathi, V., Ghosh, R. and Subramanian, S. (2017) Long non-coding RNAs regulating immunity in insects. *Noncoding RNA,* 3(1).

Scali, C., Catteruccia, F., Li, Q. and Crisanti, A. (2005) Identification of sex-specific transcripts of the *Anopheles gambiae* doublesex gene. *Journal of Experimental Biology* 208, 3701–3709.

Schember, I. and Halfon, M.S. (2021) Identification of new *Anopheles gambiae* transcriptional enhancers using a cross-species prediction approach. *Insect Molecular Biology* 30, 410–419.

Schinko, J.B., Weber, M., Viktorinova, I., Kiupakis, A., Averof, M. *et al.* (2010) Functionality of the GAL4/UAS system in *Tribolium* requires the use of endogenous core promoters. *BMC Developmental Biology* 10, 53.

Scolari, F., Schetelig, M.F., Bertin, S., Malacrida, A.R., Gasperi, G. and Wimmer, E.A. (2008) Fluorescent sperm marking to improve the fight against the pest insect *Ceratitis capitata* (Wiedemann: Dipters: Tephritidae). *Nature Biotechnology* 25(1), 76–84.

Segert, J.A., Gisselbrecht, S.S. and Bulyk, M.L. (2021) Transcriptional silencers: driving gene expression with the brakes on. *Trends in Genetics* 37, 514–527.

Sethi, S. and Wang, J.W. (2017) A versatile genetic tool for post-translational control of gene expression in *Drosophila melanogaster*. *Elife,* 6.

Shankar, S., Tauxe, G.M., Spikol, E.D., Li, M., Akbari, O.S. *et al.* (2020) Synergistic coding of human odorants in the mosquito brain. *bioRxiv* 11.02.365916.

Sharakhov, I.V. and Sharakhova, M.V. (2015) Heterochromatin, histone modifications, and nuclear architecture in disease vectors. *Current Opinion in Insect Science* 10, 110–117.

Shaul, O. (2017) How introns enhance gene expression. *International Journal of Biochemistry & Cell Biology* 91, 145–155.

Shaw, W.R., Teodori, E., Mitchell, S.N., Baldini, F., Gabrieli, P. *et al.* (2014) Mating activates the heme peroxidase HPX15 in the sperm storage organ to ensure fertility in *Anopheles gambiae*. *Proceedings of the National Academy of Sciences USA* 111, 5854–5859.

Sheng, G., Thouvenot, E., Schmucker, D., Wilson, D.S. and Desplan, C. (1997) Direct regulation of rhodopsin 1 by Pax-6/eyeless in *Drosophila*: evidence for a conserved function in photoreceptors. *Genes and Development* 11(9), 1122–1131.

Shotkoski, F., Morris, A.C., James, A.A. and ffrench-Constant, R.H. (1996) Functional analysis of a mosquito gamma-aminobutyric acid receptor gene promoter. *Gene* 168(2), 127–133.

Simoni, A., Hammond, A.M., Beaghton, A.K., Galizi, R., Taxiarchi, C. *et al.* (2020) A male-biased sex-distorter gene drive for the human malaria vector *Anopheles gambiae*. *Nature Biotechnology* 38, 1054–1060.

Smith, R.C., Walter, M.F., Hice, R.H., O'Brochta, D.A. and Atkinson, P.W. (2007) Testis-specific expression of the beta2 tubulin promoter of *Aedes aegypti* and its application as a genetic sex-separation marker. *Insect Molecular Biology* 16, 61–71.

Southall, T.D., Gold, K.S., Egger, B., Davidson, C.M., Caygill, E.E. *et al.* (2013) Cell-type-specific profiling of gene expression and chromatin binding without cell isolation: assaying RNA Pol II occupancy in neural stem cells. *Developmental Cell* 26, 101–112.

Sreenivasamurthy, S.K., Madugundu, A.K., Patil, A.H., Dey, G., Mohanty, A.K. *et al.* (2017) Mosquito-borne diseases and omics: tissue-restricted expression and alternative splicing revealed by transcriptome profiling of *Anopheles stephensi*. *OMICS* 21, 488–497.

Tallon, A.K., Hill, S.R. and Ignell, R. (2019) Sex and age modulate antennal chemosensory-related genes linked to the onset of host seeking in the yellow-fever mosquito, *Aedes aegypti. Scientific Reports* 9, 43.

Tang, W., Seth, M., Tu, S., Shen, E.Z., Li, Q. *et al.* (2018) A sex chromosome piRNA promotes robust dosage compensation and sex determination in *C. elegans*. *Developmental Cell* 44, 762–770 e3.

Taxiarchi, C., Kranjc, N., Kriezis, A., Kyrou, K., Bernardini, F. *et al.* (2019) High-resolution transcriptional profiling of *Anopheles gambiae* spermatogenesis reveals mechanisms of sex chromosome regulation. *Scientific Reports* 9, 14841.

Tazuke, S.I., Schulz, C., Gilboa, L., Fogarty, M., Mahowald, A.P. *et al.* (2002) A germline-specific gap junction protein required for survival of differentiating early germ cells. *Development* 129, 2529–2539.

Telonis-Scott, M., Kopp, A., Wayne, M.L., Nuzhdin, S.V. and Mcintyre, L.M. (2009) Sex-specific splicing in *Drosophila*: widespread occurrence, tissue specificity and evolutionary conservation. *Genetics* 181, 421–434.

Tomchaney, M., Mysore, K., Sun, L., Li, P., Emrich, S.J. *et al.* (2014) Examination of the genetic basis for sexual dimorphism in the *Aedes aegypti* (dengue vector mosquito) pupal brain. *Biology of Sex Differences* 5, 10.

Totten, D.C., Vuong, M., Litvinova, O.V., Jinwal, U.K., Gulia-Nuss, M. *et al.* (2013) Targeting gene expression to the female larval fat body of transgenic *Aedes aegypti* mosquitoes. *Insect Molecular Biology* 22, 18–30.

Trauner, J., Schinko, J., Lorenzen, M.D., Shippy, T.D., Wimmer, E.A. *et al.* (2009) Large-scale insertional mutagenesis of a coleopteran stored grain pest, the red flour beetle *Tribolium castaneum*, identifies embryonic lethal mutations and enhancer traps. *BMC Biology* 7, 73.

Travanty, E.A., Adelman, Z.N., Franz, A.W., Keene, K.M., Beaty, B.J. *et al.* (2004) Using RNA interference to develop dengue virus resistance in genetically modified *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* 34(7), 607–613.

Tsubota, T., Uchino, K., Suzuki, T.K., Tanaka, H., Kayukawa, T. *et al.* (2014) Identification of a novel strong and ubiquitous promoter/enhancer in the silkworm *Bombyx mori*. *G3: Genes, Genomes, Genetics* 4, 1347–1357.

Uchino, K., Sezutsu, H., Imamura, M., Kobayashi, I., Tatematsu, K. *et al.* (2008) Construction of a piggy-Bac-based enhancer trap system for the analysis of gene function in silkworm *Bombyx mori*. *Insect Biochemistry and Molecular Biology* 38(12), 1165–1173.

Vagin, V.V., Sigova, A., Li, C., Seitz, H., Gvozdev, V. and Zamore, P.D. (2006) A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* 313, 320–324.

Vannini, L., Augustine Dunn, W., Reed, T.W. and Willis, J.H. (2014) Changes in transcript abundance for cuticular proteins and other genes three hours after a blood meal in *Anopheles gambiae*. *Insect Biochemistry and Molecular Biology* 44, 33–43.

Vlachou, D., Schlegelmilch, T., Christophides, G.K. and Kafatos, F.C. (2005) Functional genomic analysis of midgut epithelial responses in *Anopheles* during Plasmodium invasion. *Current Biology* 15, 1185–1195.

Volohonsky, G., Terenzi, O., Soichot, J., Naujoks, D.A., Nolan, T. *et al.* (2015) Tools for *Anopheles gambiae* transgenesis. *G3: Genes, Genomes, Genetics* 5, 1151–1163.

Volohonsky, G., Hopp, A.K., Saenger, M., Soichot, J., Scholze, H. *et al.* B (2017) Transgenic expression of the anti-parasitic factor TEP1 in the malaria mosquito *Anopheles gambiae*. *PLoS Pathogens* 13, e1006113.

Wang, G.H., Gamez, S., Raban, R.R., Marshall, J.M., Alphey, L. *et al.* (2021) Combating mosquito-borne diseases using genetic control technologies. *Nature Communications* 12, 4388.

Wang, M.H., Marinotti, O., Vardo-Zalik, A., Boparai, R. and Yan, G. (2011) Genome-wide transcriptional analysis of genes associated with acute desiccation stress in *Anopheles gambiae*. *PLoS ONE* 6, e26011.

Webster, S.H., Vella, M.R. and Scott, M.J. (2020) Development and testing of a novel killer-rescue self-limiting gene drive system in *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences* 287, 20192994.

Wexler, J., Delaney, E.K., Belles, X., Schal, C., Wada-Katsumata, A. *et al.* (2019) Hemimetabolous insects elucidate the origin of sexual development via alternative splicing. *Elife* 8.

Windbichler, N., Papathanos, P.A. and Crisanti, A. (2008) Targeting the X chromosome during spermatogenesis induces Y chromosome transmission ratio distortion and early dominant embryo lethality in *Anopheles gambiae*. *PLoS Genetics* 4, e1000291.

Xu, Y., Dong, Y., Xu, Y., Lai, Z., Jin, B. *et al.* (2019) Differentiation of long non-coding RNA and mRNA expression profiles in male and female *Aedes albopictus*. *Frontiers in Genetics* 10, 975.

Ye, Z., Liu, F., Sun, H., Barker, M., Pitts, R.J. and Zwiebel, L.J. (2020) Heterogeneous expression of the ammonium transporter AgAmt in chemosensory appendages of the malaria vector, *Anopheles gambiae*. *Insect Biochemistry and Molecular Biology* 120, 103360.

Yoshida, S. and Watanabe, H. (2006) Robust salivary gland-specific transgene expression in *Anopheles stephensi* mosquito. *Insect Molecular Biology* 15, 403–410.

Zabidi, M.A. and Stark, A. (2016) Regulatory enhancer-core-promoter communication via transcription factors and cofactors. *Trends in Genetics* 32, 801–814.

Zhao, Z., Tian, D. and McBride, C.S. (2021) Development of a pan-neuronal genetic driver in *Aedes aegypti* mosquitoes. *Cell Reports Methods* 1(3), 100042.

4 RNA Interference to Modify Phenotypes in Agriculturally Important Pest and Beneficial Insects: Useful Examples and Future Challenges

Kristof De Schutter and Guy Smagghe*

Laboratory of Agrozoology, Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, 9000 Ghent, Belgium

4.1 Introduction

The discovery of RNAi brought forth a revolution in fundamental and applied research in non-model insects and enabled the next wave of revolutions through genome editing techniques. While genome editing, such as CRISPR/Cas9, is increasing in popularity, RNAi is far from obsolete. Because of its transitive silencing of gene expression, RNAi is a valuable technique to study genes crucial for the development or survival of the insect, which would be difficult with genome editing techniques.

Traditional model insect species, such as the fruit fly *Drosophila melanogaster*, have many resources in functional genomics, but they often have a low ecological context. On the other hand, for many ecologically and agriculturally important insects, the available toolset is quite small. However, the recent increase in genome and transcriptome sequence databases for non-model insects, coupled with the development of high-throughput techniques for gene expression profiling and functional characterization, has made it possible to study the biology of non-model insects. This is particularly interesting for pest insect species, where understanding the underlying mechanisms in their biology through functional genomics could lead to the development of potential pest control strategies (Vogel *et al.*, 2019; Cagliari *et al.*, 2020; Christiaens *et al.*, 2020a; Mezzetti *et al.*, 2020; Taning *et al.*, 2020).

Genome modification has long been an important tool for scientists in all fields of biology. Starting from unspecific mutagenesis with chemical mutagens and radiation, genome editing has come a long way with recent advances in protein biochemistry and genetics having provided the scientific community with powerful tools for the precise modification of genomes (Falk, 2010). These game-changing technologies, including zinc-finger nucleases (ZFNs) (Kim *et al.*, 2009), transcription activator-like effector nucleases (TALENs) (Bogdanove and Voytas, 2011) and clustered regularly interspaced short palindromic repeat/CRISPR-associated systems (CRISPR/Cas9) (Sorek *et al.*, 2013), allow a deeper understanding of the mechanisms underlying

---

*Corresponding author, email: Guy.Smagghe@UGent.be

developmental processes and diseases and have potential in the treatment of diseases.

Also, in insects, these tools offer scientists exciting opportunities for tackling public health and environmental issues, to efficiently generate novel economic insect strains and for functional genetic studies (Taning *et al.*, 2017; Gantz and Akbari, 2018; Xu *et al.*, 2019) (see Concha and Papa, Chapter 7, this volume). Despite the rapid increase in use of these genome editing tools, and especially CRISPR/Cas9, in insects, these technologies are still facing several challenges and drawbacks. For example, the complexity of design (ZFNs) and construction (TALENs) and the high production costs (ZFNs, TALENs) are major factors constraining their use (Reid and O'Brochta, 2016; Hillary *et al.*, 2020). In addition, challenges in the delivery of the constructs limit the application of these technologies to a small number of mainly (if not all) model insect species (Reid and O'Brochta, 2016; Taning *et al.*, 2017; Xu *et al.*, 2019). This is also the case for gene drive studies which have been focused on model and high-profile species such as mosquitoes (Hammond *et al.*, 2017; Kyrou *et al.*, 2018), *D. melanogaster* (Guichard *et al.*, 2019; Kandul *et al.*, 2020) and *Tribolium castaneum* (Drury *et al.*, 2017). Another obstacle of genome editing is the difficulty and labour intensity in selecting an edited insect (Xu *et al.*, 2019).

Because of these challenges, the selection of a good candidate gene is of the utmost importance. A quick and easy screening method of potential targets and interesting phenotypes could be beneficial, especially in non-model organisms. RNA interference (RNAi) is a naturally occurring gene regulation mechanism, first elucidated in 1998 (Fire *et al.*, 1998) and since then frequently used for the functional study of genes. RNAi is a highly conserved mechanism in eukaryotic organisms, in which messenger RNA (mRNA) is cleaved by the RNAi machinery, leading to inactivation of gene expression (Baum and Roberts, 2014). This process is triggered by the presence of double-stranded RNA (dsRNA) which is cleaved into small interfering RNA (siRNA) fragments by the endoribonuclease

Dicer. These fragments bind to the Argonaute protein in the RNA-induced silencing complex (RISC), after which one strand of the double-stranded siRNA is removed. The remaining strand directs the RISC complex to homologous mRNA target sequences. Once bound, the Argonaute protein can cleave the mRNA, silencing the gene expression (Fig. 4.1). Although RNAi efficiency is variable in different insect groups, growth stages and tissues (Terenius *et al.*, 2011; Scott *et al.*, 2013; Cooper *et al.*, 2019), it offers an easy and quick tool to study gene function. In addition, the transient characteristic of RNAi makes it possible to study genes essential for the development, growth and survival of the insect. Therefore, RNAi and genome editing techniques could be complementing functional genomics tools (Cagliari *et al.*, 2020).

Furthermore, RNAi technology allows the design of high-throughput screening, either by systemic screens targeting individual genes or by selection-based screens using pooled libraries of dsRNA (Echeverri and Perrimon, 2006). While the former provides the broadest screen of loss-of-function phenotypes, the latter allows study of the silencing of complete pathways. In insects, several high-throughput screens have been performed in, for example, *D. melanogaster* (DasGupta and Gonsalves, 2008; Billmann and Boutros, 2016), *T. castaneum* (Knorr *et al.*, 2013; Dönitz *et al.*, 2015; Ulrich *et al.*, 2015; Schultheis *et al.*, 2019) and *Phaedon cochleariae* (Mehlhorn *et al.*, 2021). The establishment of these screening platforms offers a tool for discovery of target genes with high potential benefit (Knorr *et al.*, 2013).

Through a series of examples, this chapter will illustrate the potential of RNAi in a wide variety of research topics, including the functional analysis of genes involved in growth, development and behaviour of the insects, the study of mechanisms of pesticide resistance and the protection of beneficial insects. In addition, RNAi is shown to be a fast, easy and high-throughput technology, complementing genome editing techniques to generate clear phenotypes and screen potential targets for genome editing application.
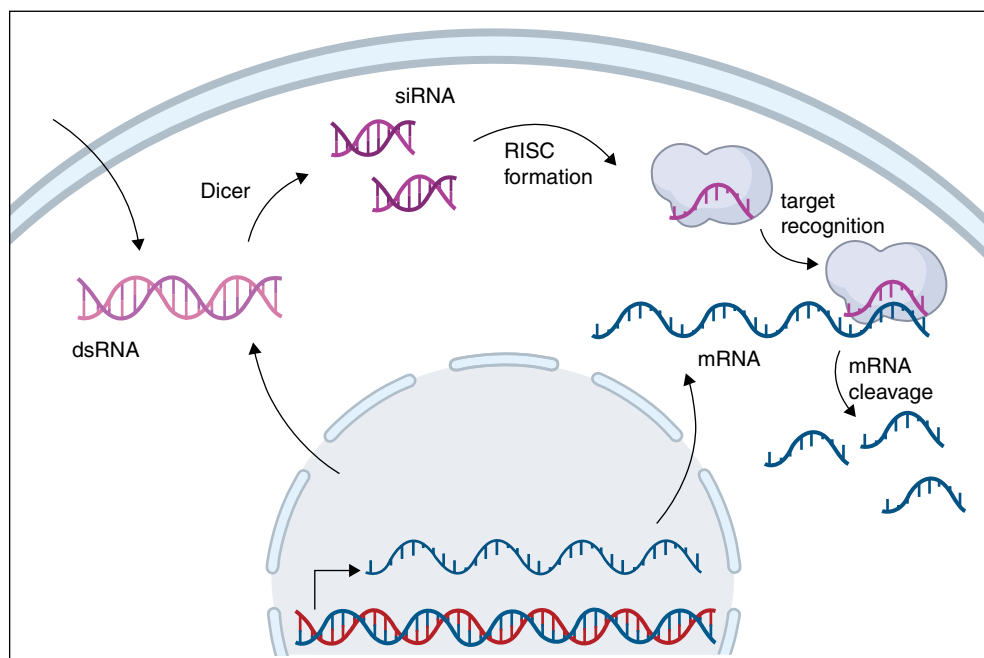
Fig. 4.1. RNAi mechanism. dsRNA, delivered by either recombinant expression or environmental application (e.g. food, injection), is processed into small interfering RNAs (siRNAs) by Dicer. These siRNAs are incorporated in the RNAi-induced silencing complex (RISC) where one of the strands is removed. The remaining strand guides the RISC complex to homologous mRNAs, which are cleaved by Argonaute. Created with Biorender.com

4.2 RNAi Phenotypes in Insect Growth, Development, Behaviour and Reproduction

RNAi has revolutionized fundamental research in insects. Before RNAi, molecular and genetic research was limited to a few model insects such as *D. melanogaster*. However, the discovery of RNAi allowed the performance of 'loss-of-function' experiments in a wide range of insects for which genetic tools and stable mutants were not available or could not easily be obtained (Bellés, 2010; Christiaens *et al.*, 2020a). While genome editing techniques will boost the next revolution of research in non-model organisms, RNAi will remain an important asset as it allows for transient knockdown of a gene (Christiaens *et al.*, 2020a). Indeed, for the investigation of crucial genes involved in insect development or reproduction, incomplete or even modulatory knockdown by

RNAi has an advantage over genome editing techniques, as the complete knockout of the gene would provide a lethal phenotype or would not produce offspring.

Over the past decade, RNAi-based functional genomics studies in non-model species have provided us with insight into the genomic and functional diversity within the insect clade (Christiaens *et al.*, 2018a). This section describes several examples of RNAi experiments, with genes of unknown function or orthologous genes described in model organisms, which reveal physiologically interesting and unique phenotypes on embryonic and post-embryonic growth, development, behaviour and reproduction (Table 4.1).

4.2.1 Growth and development

How growth and development of an organism are regulated is one of the fundamental

Table 4.1. RNA phenotypes in development, behaviour and reproduction.

	Insect	Target gene	Phenotype	Reference
Embryonal development	*E. heros*	*exd*	Complete developmental block	Cagliari *et al*., 2021
		lab	Normal development, not viable	Cagliari *et al*., 2121
		dfd	Abnormal structure on head	Cagliari *et al*., 2121
		scr	Bifurcated leg-like rostrum	Cagliari *et al*., 2121
		pb	Bifurcated leg-like rostrum	Cagliari *et al*., 2121
Post-embryonal development	*T. castaneum*	*ETHR*	Mortality, ecdysis behavioural defects	Arakane *et al*., 2008
	B. dorsalis	*ETHR-A/ETH*	Tracheal defects, failure of ecdysis	Shi *et al*., 2017
		Crz/CrzR	Delay larval pupal transition	Hou *et al*., 2017a,b
	P. citri	*Spo*	Inhibition of moulting	Li *et al*., 2017
	T. castaneum	*InR*	Reduced food uptake, lethal	Lin *et al*., 2016
		TOR	Decreased appendage growth	Lin *et al*., 2018
		FoxO	Delay of pupation	Lin *et al*., 2019
		Lac2	Cuticle sclerotization defect, lethal	Arakane *et al*., 2005
	E. heros	*awd*	Deformed and short wing	Cagliari *et al*., 2020
		th	Cuticle sclerotization defect, curved abdomen, malformed antenna and legs, lethal	Cagliari *et al*., 2021
	T. castaneum	*pgant*	Blockage of pupation	Li *et al*., 2021
		STT3/DAD1	Lethal	Walski *et al*., 2016
		GCS1/GCS2	Blockage of pupation	Walski *et al*., 2016
	N. lugens	*GCS1/GCS2*	Mortality at transition to adult	Yang *et al*., 2021
		FucT6	Moulting defect	Yang *et al*., 2021
Behaviour	*B. dorsalis*	*TRP/THRP*	Loss of aversive behaviour	Gui *et al*., 2017
	T. castaneum	*TβH*	Decreased mobility	Xu *et al*., 2018
Reproduction	*B. dorsalis*	*takeout2*	Reduced fertility and fecundity in females	Wei *et al*., 2021
		AKHR	Reduced courtship behaviour in males	Hou *et al*., 2017a,b
		ATHR-B	Decreased egg production in females	Shi *et al*., 2019

questions in biology. It starts with the embryonal development where a single cell develops into a multicellular organism, producing all of the different cells that comprise the body of the nymph or larva. The embryogenesis requires key processes, including axis formation and cell fate determination, regulated by complex and cross-communicating pathways. RNAi-mediated silencing of genes involved in these processes can lead to severe and fatal phenotypes. The study of genes involved in the embryonal development is a challenging subject and for this application transgenerational or parental RNAi could provide an interesting tool. In this variant of RNAi, the silencing phenotype is observed in the progeny of the treated parent organism (Vélez *et al.*, 2017). Parental RNAi has been explored in a range of insect species spanning different orders, including Coleoptera (Bucher *et al.*, 2002; Khajuria *et al.*, 2015; Prentice *et al.*, 2015; Vélez *et al.*, 2017) and Hemiptera (Hughes and Kaufman, 2000; Angelini *et al.*, 2005; Coleman *et al.*, 2015; Fishilevich *et al.*, 2016; Lu *et al.*, 2017; Riga *et al.*, 2019). In *Euschistus heros*, an important pest in leguminous plants in South America, parental RNAi was used to investigate the function of genes including *labial* (*lab*), *deformed* (*dfd*), *sex comb reduced* (*scr*), *extradenticle* (*exd*) and *proboscipedia* (*pb*) in rostrum development (Cagliari *et al.*, 2021). While the embryos deposited by the *lab*-silenced females developed all appendage structures, they died before hatching. In contrast, with *exd* silencing, development was halted and the embryos did not show appendage structures. Parental silencing of *dfd* caused the generation of abnormal structures between the antenna and the labium. Treatment with dsRNA targeting *scr* and *pb* caused the generation of a bifurcated rostrum with leg-like structures in the offspring (Fig. 4.2). This suggests the requirement of *scr* and *pb* in the correct determination of cell fate in the imaginal discs. The insects with the malformed rostrum were unable to feed and died shortly after hatching (Cagliari *et al.*, 2021).

After hatching, the neonate insects continue their post-embryonal development into adults. While many organisms display a gradual growth until adult, insects have a more fascinating development. As insects are enclosed in an exoskeleton, they grow in distinct stages, requiring to moult in order to grow larger. In addition, holometabolous insects undergo a complete change in physical structure during the transition from larva to adult in the process of metamorphosis. As for embryonal development, post-embryonal development of insects is regulated by complex cross-communicating pathways, requiring hormones (juvenile hormone and 20-hydroxyecdysone), neuropeptides and metabolites (Dubrovsky, 2004; Bendena, 2010; Lin and Smagghe, 2019).

Disruption of the genes involved in the neuropeptide signalling pathways, e.g. the neuropeptide receptors, regulating hormone synthesis often causes severe developmental phenotypes. For example, mutation of the *ecdysis triggering hormone receptor* (*ETHR*) in *D. melanogaster* causes high mortality and ecdysis behavioural defects (Park *et al.*, 2002) and RNAi-mediated silencing of this receptor in the model beetle *T. castaneum* results in severe deficiencies in pre-ecdysis behaviour, strongly affecting ecdysis and eclosion, ultimately leading to the death of the insect (Arakane *et al.*, 2008). Similarly, in *Bactrocera dorsalis*, an economically important pest insect of tropical and subtropical fruit, silencing of *ETHR-A* and the *ecdysis triggering hormone* (*ETH*) genes caused tracheal defects in the larvae and developmental failure at ecdysis, leading to the death of the insects (Shi *et al.*, 2017). Silencing of *Corazonin* (*Crz*), a neuropeptide hormone and neuropeptide modulator internally released within the central nerve system, and its receptor *CrzR* in *B. dorsalis* caused a delay in larval–pupal transition and pupariation (Hou *et al.*, 2017a). The accompanied delay in the expression of tyrosine hydroxylase and dopa-decarboxylase genes suggested that the inhibition of pupariation and cuticular melanization is the result of a block in dopamine synthesis (Hou *et al.*, 2017a). Also in spider mites, RNAi has contributed to the elucidation of the ecdysteroid signalling. RNAi-mediated silencing of the Halloween gene *Spook* (*Spo*) in *Panonychus citri*, an economically important and widespread pest of citrus crops, caused the inhibition of moulting which led
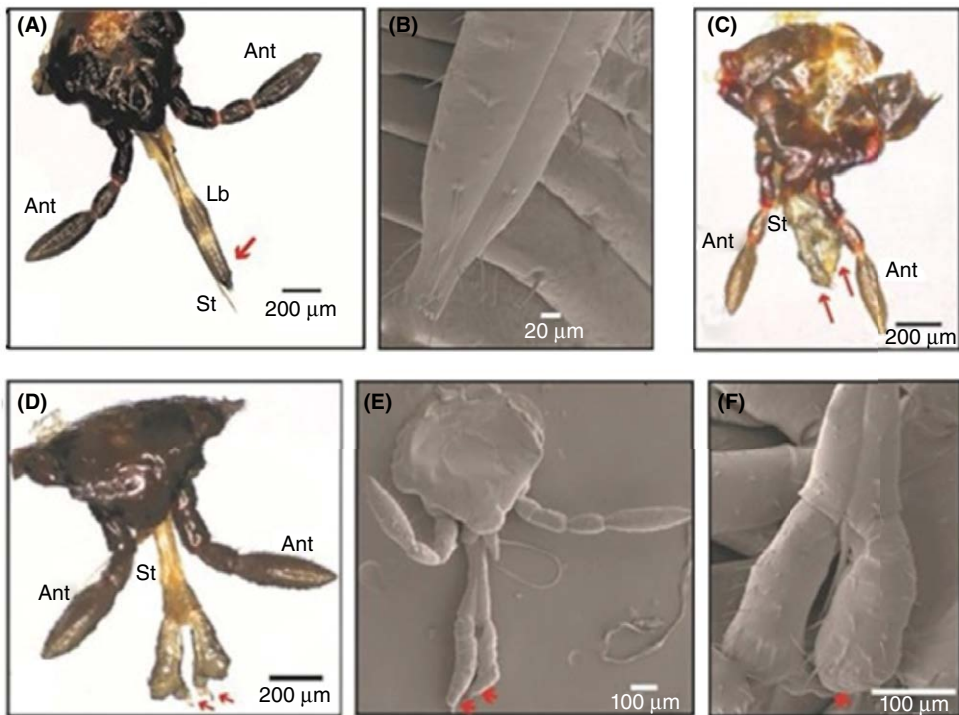
Fig. 4.2. Rostrum development in the Neotropic stinkbug *Euschistus hero*. The RNAi phenotype in nymphs from females treated with dsRNA targeting GFP **(A, B)**, sex comb reduced **(C)**, and proboscipedia **(D–F)**. **(A)** Details of the piercing/sucking mouthparts of the control treated insects (GFP). The red arrow shows the tip of the labium. **(B)** Detail of the labium tip part under the SEM. **(C)** dsScr phenotype: the labium appendage is transformed into a bifurcated rostrum with a leg-like structure, while the stylet structure is normal. **(D–F)** dsPb phenotype showing the labium appendage is transformed into a leg-like structure. On the transformed labium we can see claws (red arrows), and the stylet structure is normal as in the control. **(F)** Details of the distal part under the SEM, showing the split appendage with two leg-like structures with claws (red arrows). Ant, antenna; Lb, labium; St, stylet. Figure redrafted from Cagliari *et al.* (2021).

to the deaths of the pharate mites trapped in the old cuticle (Li *et al*., 2017). Rescue experiments revealed that while addition of 20-hydroxyecdysone (20E) could not overcome this phenotype, supplementing ponasterone A almost completely rescued the moulting defect (Li *et al*., 2017). This data suggested that ponasterone A and not 20E is the ecdysteroid hormone in mites (Grbić *et al*., 2011; Li *et al*., 2017).

Another pathway regulating growth and development is the insulin/insulin-like growth factor (IIS) and target of rapamycin (TOR) pathway (IIS/TOR). Through crosstalk with hormonal pathways, the IIS/TOR pathway regulates cell growth and differentiation, thereby controlling final adult body and organ size (Lin and Smagghe, 2019). RNAi-mediated silencing of the *insulin receptor* (*InR*) and two major components of the insulin signalling pathway, *TOR* and *FoxO*, revealed distinct phenotypes in *T. castaneum*. Silencing of *TcInR* caused a quick drop in food uptake by affecting the sulfakinin signalling pathway, resulting in lethality before pupation with a clear decrease in the weight and size of the larvae (Lin *et al*., 2016). While silencing of *TcFoxO* had an effect on the whole organism and resulted in a delay of pupation through the regulation of the ecdysteroid biosynthesis (Lin *et al*., 2018), silencing of *TcTOR* had only a moderate effect on the size of the whole organism. However, the latter had a severe effect on appendage

growth, caused by a decrease in cell size and number (Lin *et al*., 2019).

Next to the neuropeptide, hormone and insulin pathways, RNAi revealed the function of other genes in the development of insects. For example, protein glycosylation is one of the most important post-translational modifications of proteins and plays a crucial role in a variety of biological and physiological processes. RNAi allowed investigation of the role of the N- and O-glycosylation related genes in the development of insects. Interfering in the early steps of the N-glycosylation pathway leads to high mortality of the insects. For example, silencing of essential subunits of the oligosaccharyltransferase complex (*STT3* and *DAD1*) leads to high and rapid mortality of the treated insects (Walski *et al*., 2016; De Schutter *et al*., 2019). Similarly, disruption of the glucosidase activity (*GCS1* and *GCS2*) causes high mortality; however, this mortality was observed specifically at the transition to adult (Walski *et al*., 2016; Yang *et al*., 2021). Silencing of these genes, involved in the protein folding quality control, leads to a blockage of pupation in *D. melanogaster* and *T. castaneum* (Walski *et al*., 2016). Similarly, silencing of these genes in *Nilaparvata lugens* causes mortality at the transition to adult (Yang *et al*., 2021). While targeting the genes involved in the later processing steps in

T. castaneum had mild phenotypes on the wing development, larval growth and adult mobility (Walski *et al*., 2016), silencing of *Mannosidase-Ia* and the *α-1,6-Fucosyltransferase* in *N. lugens* caused high mortality, with an ecdysis phenotype in the latter case (Fig. 4.3) (Yang *et al*., 2021).

Similarly, disruption of the O-glycosylation pathway in *T. castaneum* was shown to influence the development of the insects. RNAi-mediated silencing of *N-acetylgalactosaminyltransferases* (*pgants*), required for the synthesis of the abundant core 1 O-glycans, caused a blockage of pupation, leading to high mortality in the treated insects (Li *et al*., 2021). Silencing of genes involved in the synthesis of other O-glycan structures had mild phenotypes on the adult mobility and wing formation (Li *et al*., 2021). In addition, in the Neotropical stink bug *E. heros*, silencing of the *abnormal wing disc* (*awd*) and *tyrosine hydroxylase* (*th*) genes revealed their role in the development of this insect. Disruption of the *awd* expression resulted in a distinct malformed phenotype. The wings of the adult bugs were deformed and appeared extremely shortened (Cagliari *et al*., 2020). While silencing of *awd* was not lethal, RNAi of *th* resulted in high mortality, with the insects showing defects in cuticle sclerotization, a curved abdomen and malformed antenna and legs (Cagliari *et al*., 2020). The
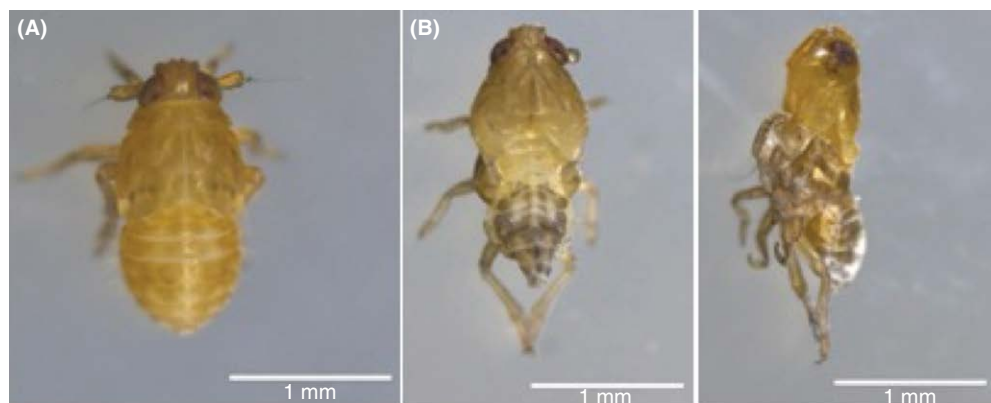
Fig. 4.3. Silencing of the *α-1,6-fucosyltransferase* gene in the brown planthopper *Nilaparvata lugens* led to a lethal phenotype with a failure of ecdysis. Following injection at 3rd nymphal instar, dsGFP-treated insects moulted normally into the 4th nymphal instar **(A)**, while dsFucT6-treated insects show high mortality during the ecdysis event **(B)**. Figure redrafted from Yang *et al*. (2021).

sclerotization, or tanning, of the cuticle involves the oxidative conjugation of proteins. Two kinds of phenoxidases, laccase and tyrosinase, have been suggested to participate in tanning (Arakane *et al.*, 2005). RNAi-mediated screening of the four phenoxidases in *T. castaneum* revealed a failure to tan in the insects treated with dsRNAs targeting *laccase 2* (*Lac2*) (Fig. 4.4). These insects were soft bodied and deformed and subsequently died, suggesting the essential role of laccase 2 in the sclerotization of larval, pupal and adult cuticles in *T. castaneum* (Arakane *et al.*, 2005).

4.2.2 Behaviour and reproduction

Insect behaviour covers a wide range of activities, including locomotion, feeding, communication, mating and various responses to environmental factors (Hoy 2019; Smagghe *et al.*, 2019). Neuropeptides are crucial in regulating myriad behavioural actions and, while these have been extensively studied, relatively little is known about most neuropeptides and only in a selected set of model insects (Schoofs *et al.*, 2017). Using RNAi, a functional analysis of genes involved in the signalling pathways leading to behavioural actions can be performed in non-model insects.

These assays have brought novel insight into olfaction, attraction and fertility in insects. For example, silencing of the *tachykinin-related peptide* (*TRP*) and its receptor (*THPR*), involved in olfactory perception, locomotion and aggression, led to a reduction in the antennal electrophysiological response in *B. dorsalis* (Gui *et al.*, 2017). In accordance with this, the dsRNA-treated flies lost their aversive behaviour to ethyl acetate, representing late-stage fermentation repulsive to flies. This confirms the role of TRP/TRPR signalling in modulating the olfactory sensitivity to avoid aversive odours (Gui *et al.*, 2017).

In *T. castaneum*, the role of tyramine β-hydroxylase (TβH) in the octopamine (OA) biosynthesis pathway was investigated using RNAi. Silencing of the gene encoding TβH caused a decrease in OA levels while resulting in an accumulation of tyramine, an intermediate in the OA synthesis pathway. This decrease of OA levels in the central nerve system led to a decrease in the mobility of the beetles (Xu *et al.*, 2018).

In *B. dorsalis*, mating success and mating duration of the adult male could be significantly reduced by the RNAi-mediated silencing of the male accessory gland-specific *takeout2* gene (Wei *et al.*, 2021). This ultimately led to a reduced fertility and fecundity of the
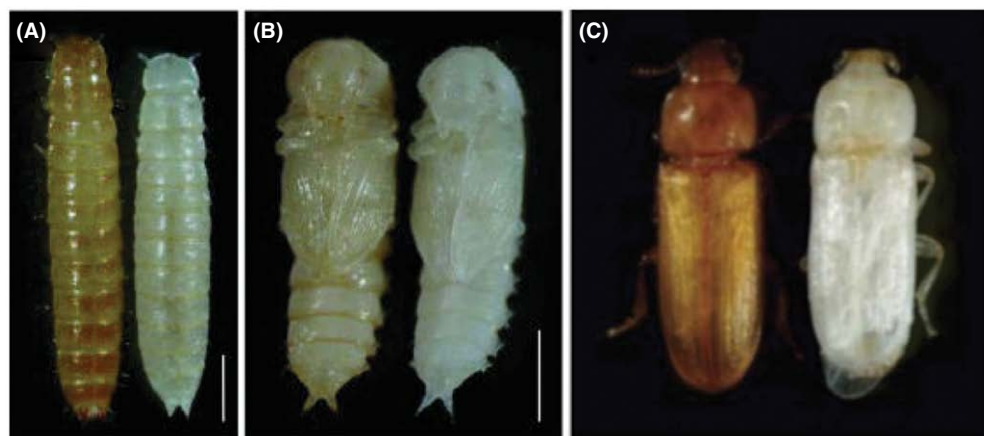
Fig. 4.4. Inhibition of cuticle tanning in the red flour beetle *Tribolium castaneum* through RNAi-mediated silencing of TcLac2. Late-stage larvae injected with dsRNA targeting Lac2 display a lack of larval **(A)** and pupal **(B)** tanning. Similarly, injection at the prepupal stage prevented tanning of the adult cuticle **(C).** In each picture, the control is shown on the left and the RNAi knockdown on the right. Size bar 1 mm. Figure redrafted from Arakane *et al.* (2005).

mated females, for which a reduction in the number of eggs laid and the hatchability of the eggs was observed (Wei *et al*., 2021). Similarly, in *D. melanogaster, takeout* genes were shown to affect courtship behaviour by processing sex-biased signals (Dauwalder *et al*., 2002). Silencing of the *B. dorsalis adipokinetic hormone receptor* (*AKHR*) had an effect on the sexual behaviour of the male flies (Hou *et al*., 2017b). Knockdown of this gene resulted in a severe decrease in courtship behaviour and tethered-flight duration of the males when starved. This phenotype could be partially rescued when re-fed, confirming the suggested dependency on the energy metabolism. In females, sexual behaviour was not affected by the RNAi, but their fecundity was decreased (Hou *et al*., 2017b). While silencing of *ATHR-A* resulted in a failure of ecdysis, RNAi of *ETHR-B* in *B. dorsalis* affects the reproduction in female adults. Silencing of this gene caused a decreased expression of the *juvenile hormone acid methyltransferase* and *Vitellogenin2* genes, leading to reduced juvenile hormone titre and egg production (Shi *et al*., 2019), a phenotype that could be rescued by co-injection of 20E (Shi *et al*., 2019).

4.3   RNAi Phenotypes Unravelling the Duality of Gene Isoforms

The presence of isoforms (highly similar proteins that originate from a single gene or gene family) can complicate functional studies. Disrupting one of the isoforms might not always give a clear phenotype, as many isoforms perform the same or similar biological roles. For example, silencing of either one of the STT3 isoforms, the catalytic subunit of the oligosaccharyl transferase complex, in *N. lugens* and *T. castaneum* only resulted in weak phenotypes, such as low mortality or minor reduction of larval growth (Walski *et al*., 2016; De Schutter *et al*., 2019). However, co-silencing of both isoforms caused a high and rapid mortality, suggesting functional redundancy between both isoforms (Walski *et al*., 2016; De Schutter *et al*., 2019). Similar observations were made in *T. castaneum* where silencing of either of the *ManI* isoforms only

caused mild phenotypes on mobility and wing formation while co-silencing of both isoforms resulted in the blockage of adult formation accompanied by high mortality (Walski *et al*., 2016).

However, the functional redundancy between both isoforms is not always bi-directional. In addition, different isoforms might have unique functions or exert their function in specific growth stages or in specific tissues. For example, silencing of *ManIa* caused high mortality in *N. lugens*, but, when targeting its isoform *ManIb*, no phenotype could be observed in the treated insects (Yang *et al*., 2021). Similarly, silencing of *ETHR-A* at larval stage caused developmental defects and mortality in *B. dorsalis*, while knockdown of its isoform *ETHR-B* had no obvious phenotypes, with nearly all insects developing successfully to the next instar (Shi *et al*., 2017). However, when treating adult *B. dorsalis* with dsRNA targeting *ETHR-B*, the fertility of the female insects is affected (Shi *et al*., 2019). These results illustrate the unique functions of isoforms depending on the developmental stage.

This observed (partial) functional redundancy between two isoforms, or the specific spatiotemporal function of isoforms, should be taken into account when selecting targets with isoforms or isoenzymes where compensation might take effect. RNAi can provide easy and fast screening to check for this redundancy before investing time and resources into creating genomic edited insects.

4.4   RNAi Phenotypes to Understand Insecticides, Mode of Action and Resistance Mechanisms

The overuse of classical pesticides in past decades has resulted in the emergence of pesticide resistance in a wide range of pest insects. RNAi offers a strategy which can be applied to identify and target genes involved in insecticide resistance. The knowledge generated through these RNAi experiments could then drive the development of gene editing strategies to overcome insecticide resistance in agricultural pests. For example,

the oriental fruit fly, *B. dorsalis*, has developed resistance to different commonly used pesticides, including malathion. It was suggested that glutathione *S*-transferases (GSTs), multifunctional enzymes that metabolize insecticides directly or indirectly, are involved in the resistance mechanism. Indeed, RNAi of *BdGSTd9* was shown to increase the toxicity of malathion in a malathion-resistant strain (Meng *et al.*, 2020). Similarly, carboxylesterases (CarEs) were suggested to be involved in metabolic resistance to organophosphate insecticides. This was confirmed through silencing of the *BdCarE2* gene, which resulted in an increased malathion susceptibility in the adult flies (Wang *et al.*, 2016). Also *T. castaneum*, a notorious pest of stored grains, has evolved resistance to all five classes of pesticides used against it. In a functional genomics and reverse genetic approach, cytochrome P450-mediated detoxification was identified as a major mechanism in the deltamethrin resistance in *T. castaneum*. Specifically, silencing of *CYP6BQ9*, a brain-specific P450 gene, was found to suppress the majority of the deltamethrin resistance in a resistant strain (Zhu *et al.*, 2010). Similarly, a cytochrome P450 (CYP6AE14) from the cotton bollworm, *Helicoverpa armigera*, was shown to be involved in larval tolerance to gossypol, a natural occurring cotton metabolite (Mao *et al.*, 2007). While RNAi-mediated silencing of *CYP6AE14* retards larval growth under control conditions, this effect is more dramatic in the presence of gossypol (Mao *et al.*, 2007).

Additionally, RNAi can be used to gain a deeper insight into the molecular mechanisms that pesticides use to exert their entomotoxicity. For example, it was found that the RNAi-mediated silencing of the immune gene *Sl102*, involved in the control of encapsulation and nodulation responses, in *Spodoptera littoralis* significantly enhances *Bacillus thuringiensis* (*Bt*)-induced insect mortality (Caccia *et al.*, 2016; Di Lelio *et al.*, 2019). This research revealed that the microbiota in the host midgut trigger a lethal septicaemia, which is enhanced by reducing the host immune responses (Caccia *et al.*, 2016), and led to the design of an agricultural application in which dsRNA-expressing bacteria are used as an immunosuppressing strategy (Caccia *et al.*, 2020). In addition, in *Plutella xylostella*, RNAi experiments showed the involvement of a midgut *trypsin* gene in the activation of the Cry1Ac protoxin, an insecticidal protein produced by *B. thuringiensis* (Gong *et al.*, 2020). As the expression of this gene was found to be reduced in resistant strains, *PxTryp_SPc1* expression was silenced in susceptible larvae. This resulted in a reduced susceptibility to Cry1Ac protoxin, confirming its involvement in the resistance and its role in the activation of the protoxin (Gong *et al.*, 2020). Similarly, the role of cadherin in *Bt*-toxicity was confirmed in *Manduca sexta* (Soberón *et al.*, 2007). RNAi-mediated silencing of the *cadherin* gene resulted in a reduced susceptibility to Cry1Ab, confirming the hypothesis that resistance to *Bt*-toxins in some insects is linked with mutations that disrupt a toxin-binding cadherin protein (Soberón *et al.*, 2007). This knowledge allowed the modification of the Cry1A toxins, by which they no longer need cadherin to form oligomers, overcoming the resistance in *M. sexta* and *Pectinophora gossypiella* (Soberón *et al.*, 2007).

These examples show the potential of RNAi to elucidate the molecular mechanism by which insecticides work and to functionally confirm the involvement of specific genes in pesticide resistance. This allows modification of toxins and the identification of promising candidates for the generation of gene drives through genome editing to eliminate pesticide resistance in pests. While resistance against classical pesticides is commonly observed, the diversity of the Dicer-generated siRNAs was believed to impede the emergence of resistance due to polymorphisms in the nucleotide sequence (Joga *et al.*, 2016). However, resistance to RNAi has been observed due to mutation of the RNAi machinery (Khajuria *et al.*, 2018; Mishra *et al.*, 2021). In *Diabrotica virgifera virgifera*, feeding on transgenic maize overexpressing dsRNA resulted in a resistance to RNAi which was localized to a single recessive locus and associated with impaired dsRNA uptake into the midgut cells (Khajuria *et al.*, 2018). Similarly, chronic exposure to increasing concentrations of non-transgenic foliar-delivered

dsRNA caused the emergence of a polygenic resistance in *Leptinotarsa decemlineata* (Mishra *et al.*, 2021). As both these mutations offered cross-resistance to other dsRNA targets, the development of potential RNAi resistance is a concern and should be studied in more detail.

4.5 RNAi Phenotypes in Crop Protection

RNAi-mediated functional screening of gene functions has allowed the identification of interesting candidates for novel pest control strategies. In contrast to the classical synthetic pesticides which have a detrimental effect on the environment, RNAi offers a novel, specific, environmentally friendly and sustainable pest management strategy. RNAi relies on a natural molecule as active ingredient and this dsRNA can be designed to selectively target specific genes in specific insects, limiting the risks of collateral damage and negative effects on non-target and beneficial organisms (Bachman *et al.*, 2016; Christiaens *et al.*, 2018b). This specificity and biodegradability of the dsRNA has provided RNAi with an immense potential for the development of novel pest control approaches. The ability of RNAi to turn off genes essential in the survival, development or reproduction of pest insects allows the creation of products with unique modes of action compared with the classical pesticides (Huvenne and Smagghe, 2010; Zotti *et al.*, 2018).

Of crucial importance in RNAi-mediated pest control strategies is the selection of the target gene. Several studies identifying suitable targets for pest control in various agricultural pest insects have been performed (Table 4.2). Because of their high sensitivity to RNAi (Zotti *et al.*, 2018), beetles are interesting insects for high-throughput screening of genes as candidates for pest control. For example, a large-scale RNAi screen in *T. castaneum* revealed novel target genes for pest control of this insect. From 5000 randomly selected genes, 11 highly efficient RNAi targets were identified, including novel targets related to the proteasome (Ulrich *et al.*,

2015). Analysis of these genes allowed the determination of gene ontology (GO)-term combinations that are predictive for RNAi target genes and this data could be useful in guiding screenings for putative efficient targets in other species (Ulrich *et al.*, 2015). Using the data from the high-throughput screening in *T. castaneum*, a two-step screening set-up was designed for *P. cochleariae*, the mustard beetle. In a first round, mortality was screened after injection of dsRNA targeting the orthologues of the *T. castaneum* genes with high RNAi efficiency. Next, the most promising targets were screened in a foliar application set-up with oral uptake of the dsRNAs (Mehlhorn *et al.*, 2021). Similarly, after demonstrating the efficiency of the RNAi response through oral application of dsRNAs in the emerald ash borer, *Agrilus planipennis*, targets for RNAi-mediated pest control were identified in this invasive forest pest insect (Rodrigues *et al.*, 2017, 2018). From an initial screen with 13 target genes, two genes showed good potential for RNAi-mediated pest control. Silencing of *shibire* (*shi*) and *heat shock protein cognate 3* (*hsp*), revealed a high mortality within one week after RNAi-mediated silencing (Rodrigues *et al.*, 2018). Also, in *D. v. virgifera*, screening the RNAi response after oral delivery of dsRNA revealed several promising genes, including *vATPase*, *αTubulin* and *Snif7*, for which silencing resulted in larval stunting and mortality (Baum *et al.*, 2007).

Next to these large-scale screenings to identify promising targets for pest control, other studies investigated the potential of individual genes. For example, in the pollen beetle *Brassicogethes aeneus*, a key pest of oilseed rape (*Brassica napus*) in Europe, a subunit of the coatomer protein complex-I involved in intracellular vesicle transport (α-COP) was selected as target for a spray-induced gene silencing (SIGS) approach through feeding of dsRNA-treated buds (Willow *et al.*, 2020). Feeding for 3 days on the treated buds caused a significant reduction in survival of the pollen beetle within 15 days (Willow *et al.*, 2020). Chronic feeding over an extended period of 17 days enhanced the RNAi efficacy and resulted in greater pest management efficacy (Willow *et al.*, 2021).

Table 4.2. RNAi phenotypes in pest control.

Target pest	Target gene	Phenotype	Reference
Agrilus planipennis	*shi/hsp*	High mortality	Rodrigues *et al*., 2018
Bactericera cockerelli	*Actin*	Decreased progeny production	Wuriyanghan and Falk, 2013
Bemisia tabaci	*TLR7*	Increased nymphal mortality	Chen *et al*., 2015
Brassicogethes aeneus	*αCOP*	High mortality	Willow *et al*., 2020
	ncm/Rop/RpIII40/dre4	Mortality	Knorr *et al*., 2018
Cimex lecturarius	*vATPase*	Mortality	Basnet and Kamble, 2018
Diabrotica virgifera virgifera	*ncm/Rop/RpIII40/dre5*	Mortality	Knorr *et al*., 2018
	vATPase/αTub/Snif7	Larval growth stunning, mortality	Baum *et al*., 2007
Diaphora citri	*Awd*	Malformed wings and increased mortality	Hajeri *et al*., 2014
Drosophila melanogaster	*RPS13/Vha26/αCOP*	Increased mortality	Taning *et al*., 2018
Helicoverpa armigera	*AK*	Drastic reduction in body weight, length and pupation rate, high mortality	Ai *et al*., 2018
Leptinotarsa decemlineata	*Actin*	Mortality	San Miguel and Scott, 2016
Lymantria dispar	*Locus365/Locus28365*	Reduction in body mass and egg masses	Ghosh and Gundersen-Rindal, 2017
Mythimna separata	*Chi*	Increased mortality and reduced body weight	Ganbaatar *et al*., 2017
Nilaparvata lugens	*Ces/CYP18A1*	Nymphal mortality	Li *et al*., 2015
	vATPase	Mortality	Chen *et al*., 2010; Liu *et al*., 2010
Ostrinia furnacalis	*KTI*	Larval mortality	Gogoi *et al*., 2017
Pectinophora gossypiella	*vATPase*	Mortality	Mohammed, 2016
Perigrinus maidis	*vATPase*	Mortality	Yao *et al*., 2013
Periplaneta fulignosa	*vATPase*	Mortality	Sato *et al*., 2019
Phaedon cochleariae	*srp54k/rop/αSNAP/hsc70-3/rpn7/rpt3*	High mortality	Mehlhorn *et al*., 2021
Phenacoccus solenopsis	*Bur/vATPase*	Physical deformities and mortality	Khan *et al*., 2018
Planococcus citri	*Actin/CHS1/vATPase*	Lower fecundity and increased mortality	Khan *et al*., 2013
Plutella xylostella	*AChE2*	60% mortality	Gong *et al*., 2013
Spodoptera exigua	*INT*	Mortality	Kim *et al*., 2015
	CHSA	Reduction in survival rates	Tian *et al*., 2009
Tribolium castaneum	*cact/srp54k/rop/αSnap/shi/pp1α-96A/ inr-a/hsc70-3/rpn7/gw/rpt3*	High mortality	Ulrich *et al*., 2015

Table redrafted from Cagliari *et al*., 2019

In this insect the use of a fusion dsRNA product, targeting both *α-COP* and *vitellogenin* (*vg*), was examined. Oral delivery of this fusion dsRNA resulted in the efficient silencing of both genes and led to high mortality of the insects (unpublished). Next to α-COP, several other genes were investigated for their potential as targets for the RNAi-mediated control of *B. aeneus*; for example, for *ncm*, *Rop*, *RpIII40* and *dre4*, RNAi-induced mortality was demonstrated after oral delivery of dsRNA (Knorr *et al.*, 2018). In addition, orthologues of these targets were shown to be effective for controlling *D. virgifera virgifera* (Knorr *et al.*, 2018). Similarly, RNAi-mediated silencing of subunits of the vacuolar protein pump, *vATPase*, was found to be a promising candidate for the control of a wide diversity of insects, including *D. virgifera virgifera* (Baum *et al.*, 2007), *N. lugens* (Chen *et al.*, 2010; Liu *et al.*, 2010), the corn planthopper *Peregrinus maidis* (Yao *et al.*, 2013), the pink bollworm *P. gossypiella* (Mohammed, 2016), the smokybrown cockroach *Periplaneta fulignosa* (Sato *et al.*, 2019) and bed bugs *Cimex lectularius* (Basnet and Kamble, 2018).

While biting/chewing insects, such as Coleoptera and Lepidoptera, are easy to target for oral delivery of dsRNAs, phloem and plant sap feeders provide a challenge for environmental RNAi. In a study with *Halyomorpha halys* and *Diaphorina citri*, two invasive hemipteran pest insects of high-value specialty crops and citrus fruits, with occurrence of pesticide resistance, different non-GMO dsRNA application methods were tested, including root drench and trunk injection (Ghosh *et al.*, 2018). Efficient silencing of *juvenile hormone acid O-methyltransferase* and *vitellogenin* was shown in these insects after feeding on plants confirmed the potential of these delivery methods (Ghosh *et al.*, 2018). However, stabilizing of the dsRNA might be needed for application in the field (Dubelman *et al.*, 2014).

4.6 RNAi Phenotypes in Beneficial Insects, Pollinators and Natural Enemies

Applications of RNAi are not limited to pest control. As a natural mechanism in the cellular defence against viruses, this technology can also be applied to protect beneficial insects (Zotti and Smagghe, 2015). About half of the leading agricultural crops, accounting for 35% of the human diet, depend on pollination by honey bees, *Apis mellifera* (Klein *et al.*, 2007). However, honey bees are facing an unusually high rate of mortality, partly because of a phenomenon known as colony collapse disorder (vanEngelsdorp *et al.*, 2007). Infections with existing and emerging pathogens have been directly or indirectly implicated in this phenomenon (Cox-Foster *et al.*, 2007). To counter the loss of these beneficial insects, RNAi-mediated strategies have been developed as preventive treatments for insect diseases. For example, feeding honey bees with a dsRNA product, Remebee-I, reduced the impact of the Israeli acute paralysis virus (IAPV), resulting in larger colony populations and increased honey production, not only under laboratory conditions but also in a field study with 160 hives in two discrete climatological and geographical locations (Hunter *et al.*, 2010). Similarly, oral administration of virus-specific dsRNA resulted in the effective repression of IAPV in bumblebees, *Bombus terrestris* (Piot *et al.*, 2015). It was observed in this study that the activation of RNAi machinery by non-specific dsRNA was also effective against IAPV (Piot *et al.*, 2015). It is interesting to mention here the development by the Monsanto company (now Bayer CropScience) of the use of RNAi through a technology called 'BioDirect'. in which dsRNA formulation is applied as a spray or syrup to the honey bees (https://www.cropscience.bayer.com/innovations/agriculture-biologicals, accessed 2 April 2022). In another approach, engineered symbiotic gut bacteria, *Snodgrassella alvi*, were used to induce RNAi-mediated immune responses in honey bees (Leonard *et al.*, 2020). On the one hand, production of (aspecific) dsRNAs by these bacteria caused the activation of immune pathway genes in the host, thereby priming the bees against infections. On the other hand, engineered bacteria, expressing dsRNA against the deformed wing virus, improved bee survival after a viral challenge (Leonard *et al.*, 2020). Similarly, *S. alvi* bacteria can be engineered to kill parasitic *Varroa* mites by triggering the mite RNAi response

(Leonard *et al.*, 2020). These results prove that symbiont-mediated RNAi approaches are powerful tools for studying bee functional genomics and safeguarding bee health (Leonard *et al.*, 2020). Similarly, engineered viruses can be used for the delivery of dsRNA in insects. This potential of virus-induced gene silencing (VIGS) was successfully shown in *D. melanogaster* cells and flies (Kolliopoulou *et al.*, 2017; Taning *et al.*, 2018).

In addition, direct feeding of dsRNAs targeting genes of honey bee-associated parasites can be employed to reduce the parasite load (Brutscher and Flenniken, 2015). For example, bees inoculated with *Nosema ceranae* spores and fed dsRNA targeting *Nosema*-specific *ADP/ATP* genes had reduced spore count, and *N. ceranae* had a lower expression of the targeted genes (Paldi *et al.*, 2010). Likewise, when bees were fed dsRNA targeting sequence-specific housekeeping genes of the mite *Varroa destructor*, the mites had lower levels of the targeted transcripts (Garbian *et al.*, 2012). In another example, dsRNA targeting *kinotoplastid membrane protein-11* was tested for its potential to control gut trypanosomes, such as *Crithidia mellificae* and *C. bombi*, in honey bees and bumblebees. Treatment with the dsRNAs resulted in a growth reduction of *C. mellificae* but not for the bumblebee parasite, as no functional *Dicer* or *Argonaut* genes were identified in *C. bombi* (de Sousa Pereira *et al.*, 2019).

However, many insect-infecting viruses have evolved specific mechanisms to counteract the RNAi-mediated antiviral defence (Brutscher and Flenniken, 2015; Li and Ding, 2006; Wu *et al.*, 2010). These include protein suppressors of RNAi (viral suppressors of RNAi, VSRs) that act by diverse mechanisms to directly block RNAi-based restrictions on viral replication (Li and Ding, 2006; Wu *et al.*, 2010; Brutscher and Flenniken, 2015). VSRs are encoded in all insect-pathogenic viruses, such as honey bee viruses (Brutscher and Flenniken, 2015). For example, the B2 protein dimer of Flock house virus binds dsRNA, subsequently preventing Dicer cleavage and siRNA loading into RISC (Chao *et al.*, 2005; Lingel *et al.*, 2005). Arbovirus infections in mosquitoes, on the other hand, are generally non-pathogenic and a robust suppression of RNAi would lead to pathogenesis and death of the vector, which would be detrimental to the virus (Myles *et al.*, 2008; Cirimotich *et al.*, 2009). No VSRs have been identified in these viruses, but these have evolved more subtle mechanisms for evading the mosquito RNAi (Blair and Olson, 2015; Liu *et al.*, 2019). These mechanisms include sequestering of the dsRNAs that trigger RNAi. For example, dengue virus infection leads to a rearrangement of intracytoplasmic membrane structures in mosquito cells to enclose dsRNA-containing replication complexes in double membrane vesicles (Junjhon *et al.*, 2014). In another strategy, unique viral RNA structures act as molecular decoys or sponges to sequester or inactivate host cell proteins required for antiviral activities (Charley and Wilusz, 2014). For example, West Nile virus sfRNA has been proposed to act as a competing substrate for Dicer (Schnettler *et al.*, 2012). These studies reveal a complex interaction between the viruses and the host RNAi mechanism (Liu *et al.*, 2019).

Natural enemies of pest insects, such as predators or parasitic wasps, are also sensitive to RNAi. For example, in *Nasonia vitripennis*, a parasitoid wasp, an efficient RNAi response was obtained after dsRNA injection (Werren *et al.*, 2009). Furthermore, four closely related species make *Nasonia* an excellent system for a variety of genetic studies and to investigate parasitoid/host dynamics, host preference and specialist versus generalist biology (Werren *et al.*, 2009). Similarly, two species of Coccinellidae, *Coccinella septempunctata* and *Adalia bipunctata*, were found to be sensitive to dietary RNAi, with *C. septempunctata* being more sensitive than *A. bipunctata* (Haller *et al.*, 2019). The zoophytophagous mirid bug *Nesidiocoris tenuis*, an efficient predator of *Tuta absoluta*, also displayed an efficient RNAi response after feeding on dsRNA targeting *Nt-αCOP* (Sarmah *et al.*, 2021). On the one hand, the possibility to generate an efficient RNAi response in these natural enemies provides a possibility for applications, but on the other hand it is a risk for off-target effects as these insects come into direct or indirect contact with the dsRNAs applied in pest control. Therefore, a thorough risk assessment is

essential before bringing RNAi-based products into the field.

4.7 RNAi in the Field: Considerations for Biosafety

The RNAi-mediated screening and functional analysis of genes for pest control have led to the development of the first commercial RNAi-based products entering the market. Using the host-induced gene silencing (HIGS) approach, a maize variety (Mon87411) was designed expressing dsRNA targeting the *Snf*7 gene of the western corn rootworm (*D. virgifera virgifera*). Another insect pest receiving considerable focus for commercial exploitation of the RNAi technology is the Colorado potato beetle, *Leptinotarsa decemlineata*, a major and widespread pest of potatoes with a high sensitivity to RNAi effects. Recently, Syngenta showed the efficiency of the company's spray-induced gene silencing (SIGS)-based product against *L. decemlineata* in a field trail, translating the RNAi-mediated insect mortality and plant protection observed in laboratory-based assays to field efficacy (Bramlett *et al*., 2020). Also GreenLight Biosciences presented data from two years of successful trials (over 20 field trials across five states in the USA) with positive performance of dsRNA to control the Colorado potato beetle (https://www.greenlightbiosciences.com/plant-and-animal-health, accessed 2 April 2022). For the protection of pollinators, RNAi-based products are moving into the market: 'BioDirect', a SIGS-based product for the control of *Varroa* in honey bees, was developed by Monsanto/Bayer showing high selectivity to *Varroa* and reducing mite levels and increasing colony survival rates (Masucci, 2019).

When applying RNAi-based products in the field, not only the target pest insect will receive the dsRNA molecules but also non-target organisms might be exposed. Therefore, the target region and dsRNA molecule design are of the essence (Cagliari *et al*., 2019). While a high degree of specificity towards the target insect has been observed in genetically modified plants (Dillin, 2003; Whyard

et al., 2009; Petrick *et al*., 2013), some studies have shown that siRNAs can lead to the silencing of non-target genes (Birmingham *et al*., 2006). For example, plant-expressed dsRNA targeting *D. virgifera virgifera vATPase* subunits *A* and *E* also caused significant mortality in *L. decemlineata* (Baum *et al*., 2007). However, a biosafety analysis with a non-target lepidopteran species, *Danaus plexippus*, revealed no effects on this organism (Pan *et al*., 2017). In contrast, other dsRNAs can be made very specific. For example, a non-target organism screening with the Syngenta dsRNA-based biocontrol product targeting *L. decemlineata* revealed the selectivity and safety of the dsRNA sequence even for closely related species and beneficial insects (Bramlett *et al*., 2020). Similarly, a genome-wide off-target screen in *B. terrestris* with dsRNA targeting pollen beetle *αCOP* revealed no reduction in the transcript level for all putative off-targets, including an off-target with a 20-continous-nt match (Taning *et al*., 2021). Off-target effects were also screened for a set of potential targets in the emerald ash borer. This analysis confirmed the specificity of the dsRNA and suggested that they are potential targets to suppress *Agrilus planipennis* populations (Rodrigues *et al*., 2018).

4.8 RNAi Future Challenges for Fundamental Mechanisms and Applications

While injection of dsRNA is an easy method to induce an RNAi response, it is not possible in a pest control strategy. For pest control, the RNAi approach can be applied *in planta*, through the production of genetically modified crops expressing the dsRNA against a target pest such as insects or mites (Niu *et al*., 2018a; Christiaens *et al*., 2020b). While this approach has been shown to be successful with one product on the market, the HIGS approach faces several challenges, including technical difficulties for transformation of crop species and a negative public perception (Christiaens *et al*., 2020b; Arpaia *et al*., 2021). These challenges have stimulated

research into the use of exogenous application of dsRNA, for example spraying of dsRNA, root drenching, seed soaking and trunk injection (Taning *et al.*, 2019) (Fig. 4.5A).

However, environmental application of the dsRNA leads to high variability in the RNAi response (Miller *et al.*, 2012). Two important factors affecting RNAi efficiency are differences in the dsRNA uptake into cells and in the stability of the dsRNAs against, for example, dsRNA-degrading enzymes (nucleases) (Zhu and Palli, 2020; De Schutter *et al.*, 2021). Owing to their large size and highly negative charge, dsRNAs cannot easily enter the cells (Whitehead *et al.*, 2009; Scott *et al.*, 2013). Although some core components are known, many questions still remain concerning the dsRNA uptake pathways. In insects, two different uptake mechanisms have been described so far: (i) a pathway based on endocytosis-mediated uptake mechanisms (Saleh *et al.*, 2006; Cappelle *et al.*, 2016); and (ii) a pathway mediated by specific dsRNA channels (Winston *et al.*, 2002). The RNAi-mediated silencing of genes putatively involved in RNAi is an elegant strategy to study the molecular mechanisms of RNAi, including dsRNA internalization. Through these so-called

RNAi-of-RNAi experiments, the contribution of two different *sid-1-like* (*sil*) genes, *silA* and *silC*, and two elements of the endocytic pathway, clathrin heavy chain and the 16kDa subunit of the vacuolar H+ATPase (vha16), to the RNAi response were demonstrated (Cappelle *et al.*, 2016). Besides cellular uptake, stability of the dsRNA is also an important factor undermining RNAi efficacy (Fig. 4.5B). The instability of dsRNA is mainly attributed to the presence of nucleases. Next to the microbial nucleases (Dubelman *et al.*, 2014), damage to the plant (during dsRNA application) can result in the release of nucleases and subsequent degradation of the exogenously applied dsRNA and, especially in insects, extracellular degradation of dsRNA by nucleases in the gut has been identified as a key factor explaining reduced RNAi efficacy (Christiaens *et al.*, 2014, 2016, 2018b; Prentice *et al.*, 2017; Guan *et al.*, 2018; Castellanos *et al.*, 2019; Ghodke *et al.*, 2019). This was confirmed through RNAi experiments in which the expression of dsRNAs was silenced. Treatment with the dsRNAs improved the oral RNAi efficacy in the southern green stinkbug *Nezara viridula* (Sharma *et al.*, 2021).
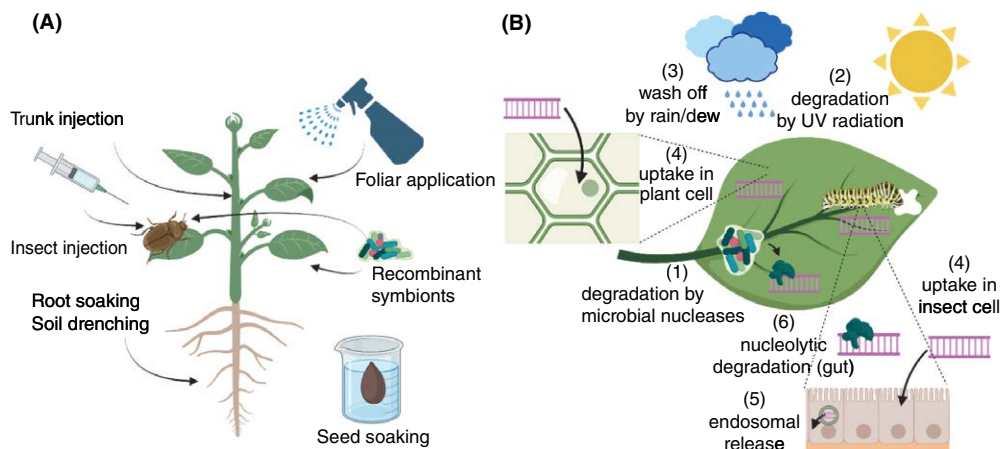
Fig. 4.5. Methods of dsRNA application in exogenous RNAi approaches (A) and the barriers it faces (B). External and internal barriers can affect the efficiency of the RNAi response. External factors include the degradation of the dsRNA by microbial nucleases (1) and UV radiation (2) and the wash-off of the applied dsRNA by rain or dew into the soil, where it is rapidly degraded by nucleases (3). Internal factors include the inefficient cellular uptake of the dsRNA (4), low endosomal release (5) and the presence of nucleases in the salivary glands, midgut and haemolymph of insects (6). Panel (B) redrafted from De Schutter *et al.* (2021). Created with Biorender.com

The use of dsRNA carrier molecules was found to increase the RNAi efficacy. These systems are designed to efficiently deliver their dsRNA cargo into the cells by avoiding RNAi barriers such as an inefficient cellular uptake, a low endosomal release and extracellular degradation of the dsRNA (De Schutter et al., 2021). The use of carrier systems has been proved to be effective to enhance RNA delivery for medical applications and the number of proof-of-concept studies to apply these in the agricultural industry is growing rapidly (Christiaens et al., 2018b; Vogel et al., 2019; De Schutter et al., 2021). Further research is needed to elucidate the molecular mechanism by which these carriers exert their function (De Schutter et al., 2021).

Additional fundamental knowledge is required on the mechanisms of RNAi. For example, systemic RNAi is an understudied aspect of RNAi, especially in insects (Zhu and Palli, 2020). Although observations suggested that a form of systemic transport must be present (Vélez and Fishilevich, 2018), the underlying mechanisms have not been elucidated. In plants, systemic RNAi is based on the RNA-dependent RNA polymerase (RdRp) and the spread of the siRNAs through the plasmodesma (Dalakouras et al., 2020). However, homologues of these RdRps have not been identified in insects (Jose and Hunter, 2007; Tomoyasu et al., 2008; Tribolium Genome Sequencing Consortium, 2008). Recently, extracellular vesicles (EVs) were shown to be (at least partially) involved in the systemic spread of RNAi in T. castaneum TcA cells (Mingels et al., 2020). Further research is needed to fully elucidate the mechanisms of systemic RNAi.

Regarding biosafety, several questions remain. Although RNAi is perceived to provide greater selectivity in pest control, some studies have shown that unintentional off-target gene silencing in target cells and in non-target organisms cannot be ruled out a priori (Qiu et al., 2005; Sigoillot et al., 2012; Haller et al., 2019). These off-target and non-target effects are difficult to predict; for example, in plants and human cell lines silencing has been observed even with some level of sequence mismatch to a processed 21–25 bp siRNA (Du et al., 2005; Liu et al., 2014). Given the small sizes of siRNAs and the 'forgiveness' of mismatches, it is not surprising that off-target binding sites could be quite common and could increase the potential for off-target gene silencing effects in non-target organisms (NTOs) (Taning et al., 2021). In insects, only very limited data exist on the minimum requirements for an siRNA sequence (length, mismatch tolerance, position of mismatch, etc.) to induce transcript knockdown. Therefore, an improved understanding of the minimum requirements for effective transcript knockdown is required, to improve our understanding on how to assess the likelihood of off-target gene silencing in the context of crop protection and environmental safety (Taning et al., 2021). Another challenge is to increase our knowledge on aspects of protein turnover and the impact of isoforms, as these are crucial to establish phenotypes by RNAi. In addition, a deeper insight into the effects of RNAi on the innate immunity of the insects is needed to understand the cross-talk between RNAi and other immune pathways (Christiaens et al., 2020a). For example, the potential fitness cost by exploiting the RNAi pathway should be investigated in insects, especially in beneficial insects that will be exposed to nonspecific dsRNA that will activate the RNAi- and immune-pathways (Wang et al., 2016, 2020). In addition, it needs to be investigated if the exposure to non-target dsRNAs has an effect on the capacity to fight off viral and other infections (Christiaens et al., 2020a).

Despite these challenges, RNAi is a promising technology for pest control and the protection of beneficial insects. In addition, RNAi-based products can be used in an integrated pest management (IPM) strategy. IPM is an ecosystem-based strategy that focuses on long-term prevention of pests or their damage through a combination of techniques such as biological control, habitat manipulation, modification of cultural practices, use of resistant varieties and targeted and restricted use of pesticides. Because of the different mode of action, RNAi can complement the use of classical pesticides. For example, for the control of Varroa in honey bees, combining the RNAi-based treatment

(BioDirect) with the classical chemical treatment Apivar enhances the control of these parasitic mites (Masucci, 2019). In addition, due to the selectivity of the dsRNA, RNAi can be used in combination with natural predators. For example, dsRNA targeting the *αCOP* gene in *Tuta absoluta* has no lethal or sub-lethal effects on its predator *N. tenuis* (Sarmah *et al*., 2021). This indicates the compatibility of this biocontrol agent along with RNAi-mediated management in order to suppress *T. absoluta* efficiently in tomato crops (Sarmah *et al*., 2021). In an alternative approach, silencing of the *gram-negative binding protein 1* gene in *Acyrthosiphon pisum*, *Myzus persicae* and *Aphis citricidus* decreased the activity of immune-related phenyloxidase (Ye *et al*., 2021). This leads to an increase in the virulence of the fungus *Beauveria bassiana*, used as a biological control agent for aphids. In addition, no negative effects of the dsRNA of *B. bassiana* treatment were observed in the aphid predator *Propylaea japonica*, suggesting that RNAi can be combined with entomopathogenic fungi and ladybeetle predators (Ye *et al*., 2021). The latter is a good recent example of how RNAi phenotypes can contribute to a greener and safer agriculture production, combining RNAi and biological control in the context of IPM (Niu *et al*., 2018b).

As RNAi-based approaches start making a contribution towards IPM and sustainable agriculture and as literature on RNAi-based control in crop protection continues to expand, the European 'iPlanta' Cooperation in Science and Technology (COST) action, CA15223 'Modifying plants to produce interfering RNA' (available at https://iplanta.univpm.it, accessed 2 April 2022), with a focus on RNAi in agriculture, has suggested it is timely to evaluate both the trends and influence of its development and to provide an indication of the research and development landscape, the prolific centres of research and their collaborations (Mezzetti *et al*., 2020). This COST action is a multi-actor platform of excellence in RNAi mechanisms, applications, biosafety, socio-economic issues and communication in many EU and nearby countries, and cooperating researchers in associated countries in the Americas, Australia and Asia.

4.9 Conclusions

While genome editing techniques are driving the next revolution in fundamental and applied research in non-model insects, the advantages of RNAi will ensure that RNAi remains an important tool. Not only does RNAi allow for fast, easy and high-throughput screening of potential targets for genome editing, but also the transitive knockdown of gene expression allows the investigation of genes that are crucial for the development or the survival of the insect, which would be difficult with genome editing techniques. In addition, RNAi can be applied in a non-GMO strategy.

The knowledge generated through RNAi experiments, not only on targets for pest control, but also on the mechanisms of pesticide resistance and on its application for the protection of beneficial insects, can be used to drive novel solutions and strategies using genome editing and gene drive techniques. In conclusion, RNAi and genome editing technologies can be complementary, supporting and enhancing both technologies.

Acknowledgements

References

Ai, X., Wei, Y., Huang, L., Zhao, J., Wang, Y. *et al.* (2018) Developmental control of *Helicoverpa armigera* by ingestion of bacteria expressing dsRNA targeting an arginine kinase gene. *Biocontrol Science and Technology* 28, 253–267.

Angelini, D.R., Liu, P.Z., Hughes, C.L. and Kaufman, T.C. (2005) Hox gene function and interaction in the milkweed bug *Oncopeltus fasciatus* (Hemiptera). *Developmental Biology* 287, 440–455.

Arakane, Y., Muthukrishnan, S., Beeman, R.W., Kanost, M.R. and Kramer, K.J. (2005) Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. *Proceedings of the National Academy of Sciences of the United States of America* 102, 11337–11342.

Arakane, Y., Li, B., Muthukrishnan, S., Beeman, R.W., Kramer, K.J. *et al.* (2008) Functional analysis of four neuropeptides, EH, ETH, CCAP and bursicon, and their receptors in adult ecdysis behavior of the red flour beetle, *Tribolium castaneum*. *Mechanisms of Development* 125, 984–995.

Arpaia, S., Smagghe, G. and Sweet, J.B. (2021) Biosafety of bee pollinators in genetically modified agro-ecosystems: current approach and further development in the EU. *Pest Management Science* 77, 2659–2666.

Bachman, P.M., Huizinga, K.M., Jensen, P.D., Mueller, G., Tan, J. *et al.* (2016) Ecological risk assessment for DvSnf7 RNA: a plant-incorporated protectant with targeted activity against western corn rootworm. *Regulatory Toxicology and Pharmacology* 81, 77–88.

Basnet, S. and Kamble, S.T. (2018) RNAi-mediated knockdown of vATPase subunits affects survival and reproduction of bed bugs (Hemiptera: Cimicidae). *Journal of Medical Entomology* 55, 540–546.

Baum, J.A, Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P. *et al.* (2007) Control of coleopteran insect pests through RNA interference. *Nature Biotechnology* 25, 1322–1326.

Baum, J.A. and Roberts, J.K. (2014) Progress towards RNAi-mediated insect pest management. *Advances in Insect Physiology* 47, 249–295.

Bellés, X. (2010) Beyond *Drosophila*: RNAi in vivo and functional genomics in insects. *Annual Review of Entomology* 55, 111–128.

Bendena, W.G. (2010) Neuropeptide physiology in insects. *Advances in Experimental Medical Biology* 692, 166–191.

Billmann, M. and Boutros, M. (2016) Methods for high-throughput RNAi screening in Drosophila cells. *Methods in Molecular Biology* 1478, 95–116.

Birmingham, A., Anderson, E.M., Reynolds, A., Ilsley-Tyree, D., Leake, D. *et al.* (2006) 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nature Methods* 3, 199–204.

Blair, C.D. and Olson, K.E. (2015) The role of RNA interference (RNAi) in Arbovirus–vector interactions. *Viruses* 7, 820–843.

Bogdanove, A.J. and Voytas, D.F. (2011) TAL effectors: customizable proteins for DNA targeting. *Science* 333, 1843–1846.

Bramlett, M., Plaetinck, G. and Maienfrisch, P. (2020) RNA-based biocontrols – a new paradigm in crop protection. *Engineering* 6, 522–527.

Brutscher, L. and Flenniken, M.L. (2015) RNAi and antiviral defense in the honey bee. *Journal of Immunology Research* 2015, 941897.

Bucher, G., Scholten, J. and Klingler, M. (2002) Parental RNAi in *Tribolium* (Coleoptera). *Current Biology* 12, 85–86.

Caccia, S., Di Lelio, I., La Storia, A., Marinelli, A., Varricchio, P. *et al.* (2016) Midgut microbiota and host immunocompetence underlie *Bacillus thuringiensis* killing mechanism. *Proceedings of the National Academy of Sciences USA* 113, 9486–9491.

Caccia, S., Astarita, F., Barra, E., Di Lelio, I., Varricchio, P. *et al.* (2020) Enhancement of *Bacillus thuringiensis* toxicity by feeding *Spodoptera littoralis* larvae with bacteria expressing immune suppressive dsRNA. *Journal of Pest Science* 93, 303–314.

Cagliari, D., Dias, N.P., Galdeano, D.M., dos Santos, E.A., Smagghe, G. *et al.* (2019) Management of pest insects and plant diseases by non-transformative RNAi. *Frontiers in Plant Science* 10, 1319.

Cagliari, D., Smagghe, G., Zotti, M. and Taning, C.N.T. (2020) RNAi and CRISPR/Cas9 as functional genomics tools in the neotropical stink bug, *Euschistus heros. Insects* 11, 838.

Cagliari, D., Taning, C.N.T., Christiaens, O., De Schutter, K., Lewille, B. *et al.* (2021) Parental RNA interference as a tool to study genes involved in rostrum development in the Neotropical brown stink bug, *Euschistus heros*. *Journal of Insect Physiology* 128(104161), 1–7.

Cappelle, K., de Oliveira, C.F.R., Van Eynde, B., Christiaens, O. and Smagghe, G. (2016) The involvement of clathrin-mediated endocytosis and two Sid-1-like transmembrane proteins in double-stranded RNA uptake in the Colorado potato beetle midgut. *Insect Molecular Biology* 25, 315–323.

Castellanos, L.N., Smagghe, G., Sharma, R., Oliveira, E.E. and Christiaens, O. (2019) Liposome encapsulation and EDTA formulation of dsRNA targeting essential genes increase oral RNAi-caused mortality in the Neotropical stink bug *Euschistus heros*. *Pest Management Science* 75, 537–548.

Chao, J.A., June, H.L., Chapados, B.R., Debler, E.W., Schneemann, A. *et al.* (2005) Dual modes of RNA silencing suppression by Flock House virus protein B2. *Nature Structural & Molecular Biology* 12, 952–957.

Charley, P.A. and Wilusz, J. (2014) Sponging of cellular proteins by viral RNAs. *Current Opinion in Virology* 9, 14–18.

Chen, J., Zhang, D., Yao, Q., Zhang, J., Dong, X. *et al.* (2010) Feeding-based RNA interference of a trehalose phosphate synthase gene in the brown planthopper, *Nilaparvata lugens*. *Insect Molecular Biology* 19, 777–786.

Chen, X., Li, L., Hu, Q., Zhang, B., Wu, W. *et al.* (2015) Expression of dsRNA in recombinant Isaria fumosorosea strain targets the TLR7 gene in *Bemisia tabaci*. *BMC Biotechnology* 15, 64.

Christiaens, O., Swevers, L. and Smagghe, G. (2014) DsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. *Peptides* 53, 307–314.

Christiaens, O., Prentice, K., Pertry, I., Ghislain, M., Bailey, A. *et al.* (2016) RNA interference: a promising biopesticide strategy against the African sweet potato weevil *Cylas brunneus*. *Scientific Reports* 6, 38836.

Christiaens, O., Dzhambazova, T., Kostov, K., Arpaia, S., Joga, M.R. *et al.* (2018a) Literature review of baseline information on RNAi to support the environmental risk assessment of RNAi-based GM plants. *EFSA Supporting Publications* 15, 1424E.

Christiaens, O., Tardajos, M.G., Martinez Reyna, Z.L., Dash, M., Dubruel, P. *et al.* (2018b) Increased RNAi efficacy in *Spodoptera exigua* via the formulation of dsRNA with guanylated polymers. *Frontiers in Physiology* 9, 316.

Christiaens O., Niu, J. and Taning C.N.T. (2020a) RNAi in insects: a revolution in fundamental research and pest control applications. *Insects* 11, 415.

Christiaens, O., Whyard, S., Vélez, A.M. and Smagghe, G. (2020b) Double-stranded RNA technology to control insect pests: current status and challenges. *Frontiers in Plant Science* 11, 451.

Cirimotich, C.M., Scott, J.C., Phillips, A.T., Geiss, B.J. and Olson, K.E. (2009) Suppression of RNA interference increases alphavirus replication and virus-associated mortality in *Aedes aegypti* mosquitoes. *BMC Microbiology* 9, 49.

Coleman, A.D., Wouters, R.H.M., Mugford, S.T. and Hogenhout, S.A. (2015) Persistence and transgenerational effect of plant-mediated RNAi in aphids. *Journal of Experimental Botany* 66, 541–548.

Cooper, A.M., Silver, K., Zhang, J., Park, Y. and Zhu, K.Y. (2019) Molecular mechanisms influencing efficiency of RNA interference in insects. *Pest Management Science* 75, 18–28.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D. *et al.* (2007) A metagenomic survey of microbes in honey bee colony collapse disorder. *Science* 318, 283–287.

Dalakouras, A., Wassenegger, M., Dadami, E., Ganopoulos, I., Pappas, M.L. *et al.* (2020) GMO-free RNAi: exogenous application of RNA molecules in plants. *Plant Physiology* 182, 38–50.

DasGupta, R. and Gonsalves, F.C. (2008) High-throughput RNAi screen in *Drosophila*. *Methods in Molecular Biology* 469, 163–184.

Dauwalder, B., Tsujimoto, S., Moss, J. and Mattox, W. (2002) The Drosophila *takeout* gene is regulated by the somatic sex-determination pathway and affects male courtship behavior. *Genes & Development* 16, 2879–2892.

De Schutter, K., Chen, P., Shen, Y., Van Damme, E.J.M. and Smagghe, G. (2019) The OST-complex as target for RNAi-based pest control in *Nilaparvata lugens*. *Archives of Insect Biochemistry and Physiology* 101, e21555.

De Schutter, K., Christiaens, O., Taning, C.N.T. and Smagghe, G. (2021) Boosting dsRNA delivery in plant and insect cells with peptide- and polymer-based carriers: cases-based current status and future perspectives. In: Mezzetti, B., Sweet, J. and Burgos, L. (eds) *RNAi for Plant Improvement and Protection*. CAB International, Wallingford, UK, pp. 102–116.

de Sousa Pereira, K., Piot, N., Smagghe, G. and Meeus, I. (2019) Double-stranded RNA reduces growth rates of the gut parasite *Crithidia mellificae*. *Parasitology Research* 118, 715–21.

Di Lelio, I., Illiano, A., Astarita, F., Gianfranceschi, L., Horner, D. *et al.* (2019) Evolution of an insect immune barrier through horizontal gene transfer mediated by a parasitic wasp. *PLoS Genetics* 15, e1007998.

Dillin, A. (2003) The specifics of small interfering RNA specificity. *Proceedings of the National Academy of Sciences USA*, 100, 6289–6291.

Dönitz, J., Schmitt-Engel, C., Grossmann, D., Gerischer, L., Tech, M. *et al.* (2015) iBeetle-Base: a database for RNAi phenotypes in the red flour beetle *Tribolium castaneum*. *Nucleic Acids Research* 43 (Database issue), D720–725.

Drury, D.W., Dapper, A.L., Siniard, D.J., Zentner, G.E. and Wade, M.J. (2017) CRISPR/Cas9 gene drives in genetically variable and nonrandomly mating wild populations. *Science Advances* 19, E1601910.

Du, Q., Thonberg, H., Wang, J., Wahlestedt, C. and Liang, Z. (2005) A systematic analysis of the silencing effects of an active siRNA at all single-nucleotide mismatched target sites. *Nucleic Acids Research* 33, 1671–1677.

Dubelman, S., Fischer, J., Zapata, F., Huizinga, K., Jiang, C. *et al.* (2014) Environmental fate of double-stranded RNA in agricultural soils. *PLoS ONE* 9, e93155.

Dubrovsky, E.B. (2004) Hormonal cross talk in insect development. *Trends in Endocrinology & Metabolism* 16, 6–11.

Echeverri, C. and Perrimon, N. (2006) High-throughput RNAi screening in cultured cells: a user's guide. *Nature Reviews in Genetics* 7, 373–384.

Falk, R. (2010) Mutagenesis as a genetic research strategy. *Genetics* 185, 1135–1139.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. *et al.* (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811.

Fishilevich, E., Vélez, A.M., Khajuria, C., Frey, M.L.F., Hamm, R.L. *et al.* (2016) Use of chromatin remodeling ATPases as RNAi targets for parental control of western corn rootworm (*Diabrotica virgifera virgifera*) and Neotropical brown stink bug (*Euschistus heros*). *Insect Biochemistry and Molecular Biology* 71, 58–71.

Ganbaatar, O., Cao, B., Zhang, Y., Bao, D., Bao, W. *et al.* (2017) Knockdown of *Mythimna separata* chitinase genes via bacterial expression and oral delivery of RNAi effectors. *BMC Biotechnology* 17, 9.

Gantz, V.M. and Akbari, O.S. (2018) Gene editing technologies and applications for insects. *Current Opinion in Insect Science* 28, 66–72.

Garbian, Y., Maori, E., Kalev, H., Shafir, S. and Sela, I. (2012) Bidirectional transfer of RNAi between honey bee and *Varroa destructor*: *Varroa* gene silencing reduces *Varroa* population. *PLoS Pathogens* 8, e1003035.

Ghodke, A.B., Good, R.T., Golz, J.F., Russell, D.A., Edwards, O. *et al.* (2019) Extracellular endonucleases in the midgut of *Myzus persicae* may limit the efficacy of orally delivered RNAi. *Scientific Reports* 9, 11898.

Ghosh, S.K.B. and Gundersen-Rindal, D.E. (2017) Double strand RNA-mediated RNA interference through feeding in larval gypsy moth, *Lymantria dispar* (Lepidoptera: Erebidae). *European Journal of Entomology* 114, 170–178.

Ghosh, S.K.B., Hunter, W.B., Park, A.L. and Gundersen-Rindal, D.E. (2018) Double-stranded RNA oral delivery methods to induce RNA interference in phloem and plant-sap-feeding hemipteran insects. *Journal of Visual Experiments* 135, e57390.

Gogoi, A., Sarmah, N., Kaldis, A., Perdikis, D. and Voloudakis, A. (2017) Plant insects and mites uptake double-stranded RNA upon its exogenous application on tomato leaves. *Planta* 246, 1233–1241.

Gong, L., Chen, Y., Hu, Z. and Hu, M. (2013) Testing insecticidal activity of novel chemically synthesized siRNA against *Plutella xylostella* under laboratory and field conditions. *PLoS ONE* 8, e62990.

Gong, L., Kang, S., Zhou, J., Sun, D., Guo, L. *et al.* (2020) Reduced expression of a novel midgut trypsin gene involved in protoxin activation correlates with Cry1Ac resistance in a laboratory-selected strain of *Plutella xylostella* (L.). *Toxins* 12, 76.

Grbić, M., Van Leeuwen, T., Clark, R., Rombauts, S., Rouzé, P. *et al.* (2011) The genome of *Tetranychus urticae* reveals herbivorous pest adaptations. *Nature* 479, 487–492.

Guan, R.B., Li, H.C., Fan, Y.J., Hu, S.R., Christiaens, O. *et al.* (2018) A nuclease specific to lepidopteran insects suppresses RNAi. *Journal of Biological Chemistry* 293, 6011–6021.

Gui, S.-H., Jiang, H.-B., Xu, L., Pei, Y.-X., Liu, X.-Q. *et al.* (2017) Role of a tachykinin-related peptide and its receptor in modulating the olfactory sensitivity in the oriental fruit fly, *Bactrocera dorsalis* (Hendel). *Insect Biochemistry and Molecular Biology* 80, 71–78.

Guichard, A., Haque, T., Bobik, M., Xu, X.-R.S., Klanseck, C. *et al.* (2019) Efficient allelic-drive in *Drosophila*. *Nature Communications* 10, 1–10.

Hajeri, S., Killiny, N., El-Mohtar, C., Dawson, W.O. and Gowda, S. (2014) Citrus tristeza virus-based RNAi in citrus plants induces gene silencing in *Diaphorina citri*, a phloem-sap sucking insect vector of citrus greening disease (Huanglongbing). *Journal of Biotechnology* 176, 42–49.

Haller, S., Widmer, F., Siegfried, B.D., Zhuo, X. and Romeins, J. (2019) Responses of two ladybird beetle species (Coleoptera: Coccinellidae) to dietary RNAi. *Pest Management Science* 75, 2652–2662.

Hammond, A.M., Kyrou, K., Bruttini, M., North, A., Galizi, R. *et al.* (2017) The creation and selection of mutations resistant to a gene drive over multiple generations in the malaria mosquito. *PLoS Genetics* 1(3), e1007039.

Hillary, V.E., Ceasar, S.A and Ignacimuthu, S. (2020) Genome engineering in insects: focus on the CRISPR/ Cas9 system. In: Singh, V. and Dhar, P.K. (eds) *Genome Engineering via CRISPR-Cas9 System*. Academic Press, Cambridge, Massachusetts.

Hou, Q.-L., Jiang, H.-B., Gui, S.-H., Chen, E.-H., Wei, D.-D. *et al.* (2017a) A role of corazonin receptor in larval–pupal transition and pupariation in the oriental fruit fly *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae). *Frontiers in Physiology* 8, 8511406.

Hou, Q.-L., Chen, E.-H., Jiang, H.-B., Wei, D.-D., Gui, S.-H., *et al.* (2017b) Adipokinetic hormone receptor gene identification and its role in triacylglycerol mobilization and sexual behavior in the oriental fruit fly (*Bactrocera dorsalis*). *Insect Biochemistry and Molecular Biology* 90, 1–13.

Hoy, M.A. (2019) Molecular genetics of insect behavior. In: Hoy, M.A. (ed.) *Insect Molecular Genetics*. Academic Press, Cambridge, Massachusetts.

Hughes, C.L. and Kaufman, T.C. (2000) RNAi analysis of *deformed*, *proboscipedia* and *sex combs reduced* in the milkweed bug *Oncopeltus fasciatus*: novel roles for Hox genes in the Hemipteran head. *Development* 127, 3683–3694.

Hunter, W., Ellis, J., vanEngelsdorp, D., Hayes, J., Westervelt, D. *et al.* (2010) Large-scale field application of RNAi technology reducing Israeli Acute Paralysis Virus disease in honey bees (*Apis mellifera*, Hymenoptera: Apidae). *PLoS Pathogens* 6, e1001160.

Huvenne, H. and Smagghe, G. (2010) Mechanisms of dsRNA uptake in insects and potentials of RNAi for pest control: a review. *Journal of Insect Physiology* 56, 227–235.

Joga, M.R., Zotti, M.J., Smagghe, G. and Christiaens, O. (2016) RNAi efficiency, systemic properties, and novel delivery methods for pest insect control: what we know so far. *Frontiers in Physiology* 7, 553.

Jose, A.M. and Hunter, C.P. (2007) Transport of sequence-specific RNA interference information between cells. *Annual Review of Genetics* 41, 305–330.

Junjhon, J., Pennington, J.G., Edwards, T.J., Perera, R., Lanman, J. *et al.* (2014) Ultrastructural characterization and three-dimensional architecture of replication sites in dengue virus-infected mosquito cells. *Journal of Virology* 88, 4687–4697.

Kandul, N.P., Liu, J., Buchman, A., Gantz, V.M., Bier, E. *et al.* (2020) Assessment of a split homing based gene drive for efficient knockout of multiple genes. *G3: Genes, Genomes, Genetics* 10, 827–837.

Khajuria, C., Ivashuta, S., Wiggins, E., Flagel, L., Moar, W. *et al.* (2018) Development and characterization of the first dsRNA-resistant insect population from western corn rootworm, *Diabrotica virgifera virgifera LeConte*. *PLoS ONE* 13, e0194059.

Khajuria, C., Vélez, A.M., Rangasamy, M., Wang, H., Fishilevich, E. *et al.* (2015) Parental RNA interference of genes involved in embryonic development of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. *Insect Biochemistry and Molecular Biology* 63, 54–62.

Khan, A.M., Ashfaq, M., Kiss, Z., Khan, A.A., Mansoor, S. *et al.* (2013) Use of recombinant tobacco mosaic virus to achieve RNA interference in plants against the citrus mealybug, *Planococcus citri* (Hemiptera: Pseudococcidae). *PLoS ONE* 8, e73657.

Khan, A.M., Ashfaq, M., Khan, A.A., Naseem, M.T. and Mansoor, S. (2018) Evaluation of potential RNA-interference-target genes to control cotton mealybug, *Phenacoccus solenopsis* (Hemiptera: Pseudococcuidae). *Insect Science* 25, 778–786

Kim, E., Park, Y. and Kim, Y. (2015) A transformed bacterium expressing double-stranded RNA specific to integrin β1 enhances *Bt* toxin efficacy against a polyphagous insect pest, *Spodoptera exigua*. *PLoS ONE* 10, e0132631.

Kim, H.J., Lee, H.J., Kim, H., Cho, S.W. and Kim, J.S. (2009) Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. *Genome Research* 19, 1279–1288.

Klein, A.M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A. *et al.* (2007) Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society London Series B, Biological Sciences* 274, 303–313.

Knorr, E., Bingsohn, L., Kanost, M.R. and Vilcinskas, A. (2013) *Tribolium castaneum* as a model for high-throughput RNAi screening. *Advances in Biochemical Engineering and Biotechnology* 136, 163–178.

Knorr, E., Fishilevich, E., Tenbusch, L., Frey, M.L.F., Rangasamy, M. *et al.* (2018) Gene silencing in *Tribolium castaneum* as a tool for targeted identification of candidate RNAi targets in crop pests. *Scientific Reports* 8, 2061.

Kolliopoulou, A., Taning, C.N.T., Smagghe, G. and Swevers, L. (2017) Viral delivery of dsRNA for control of insect agricultural pests and vectors of human disease: prospects and challenges. *Frontiers in Physiology* 8, 399.

Kyrou, K., Hammond, A., Galizi, R., Kranjc, N., Burt, A. *et al.* (2018) A CRISPR-Cas9 gene drive targeting *doublesex* causes complete population suppression in caged *Anopheles gambiae* mosquitoes. *Nature Biotechnology* 36, 1062–1066.

Leonard, S.P., Powell, E., Perutka, J., Geng, P., Heckmann, L.C. *et al.* (2020) Engineered symbionts activate honey bee immunity and limit pathogens. *Science* 367, 573–576.

Li, F. and Ding, S.W. (2006) Virus counterdefense: diverse strategies for evading the RNA-silencing immunity. *Annual Reviews in Microbiology* 60, 503–531.

Li, G., Niu, J.Z., Zotti, M., Sun, Q.Z., Zhu, L. *et al.* (2017) Characterization and expression patterns of key ecdysteroid biosynthesis and signaling genes in a spider mite (*Panonychus citri*). *Insect Biochemistry and Molecular Biology* 87, 136–146.

Li, H., Guan, R., Guo, H. and Miao, X. (2015) New insights into an RNAi approach for plant defence against piercing-sucking and stem-borer insect pests: plant roots mediated dsRNA absorbing system. *Plant Cell and Environment* 38, 2277–2285.

Li, W., De Schutter, K., Van Damme, E.J.M. and Smagghe, G. (2021) RNAi-mediated silencing of Pgants shows core 1 O-glycans are required for pupation in *Tribolium castaneum*. *Frontiers in Physiology* 12, 629682.

Lin, X. and Smagghe, G. (2019) Roles of the insulin signaling pathway in insect development and organ growth. *Peptides* 122.

Lin, X., Yu, N. and Smagghe, G. (2016) Insulin receptor regulates food intake through sulfakinin signaling in the red flour beetle, *Tribolium castaneum*. *Peptides* 80, 89–95.

Lin, X., Yu, N. and Smagghe, G. (2018) FoxO mediates the timing of pupation through regulating ecdysteroid biosynthesis in the red flour beetle, *Tribolium castaneum*. *General and Comparative Endocrinology* 258, 149–156.

Lin, X., De Schutter, K., Chafino, S., Franch-Marro, X., Martin, D. *et al.* (2019) Target of rapamycin (TOR) determines appendage size during pupa formation of the red flour beetle *Tribolium castaneum*. *Journal of Insect Physiology* 117, 103902.

Lingel, A., Simon, B., Izaurralde, E. and Sattler, M. (2005) The structure of the flock house virus B2 protein, a viral suppressor of RNA interference, shows a novel mode of double-stranded RNA recognition. *EMBO Reports* 6, 1149–1155.

Liu, J., Swevers, L., Kolliopoulou, A. and Smagghe, G. (2019) Arboviruses and the challenge to establish systemic and persistent infections in competent mosquito vectors: the interaction with the RNAi mechanism. *Frontiers in Physiology* 10, 890.

Liu, Q., Wang, F., Axtell, M.J. (2014) Analysis of complementarity requirements for plant microRNA targeting using a *Nicotiana benthamiana* quantitative transient assay. *The Plant Cell* 26, 741–753.

Liu, S., Ding, Z., Zhang, C., Yang, B. and Liu, Z. (2010) Gene knockdown by intro-thoracic injection of double-stranded RNA in the brown planthopper, *Nilaparvata lugens*. *Insect Biochemistry and Molecular Biology* 40, 666–671.

Lu, Y., Chen, M., Reding, K. and Pick, L. (2017) Pick establishment of molecular genetic approaches to study gene expression and function in an invasive hemipteran, *Halyomorpha halys*. *Evodevo* 8, 1–11.

Mao, Y.-B., Cai, W.-J., Wang, J.-W., Hong, G.-J., Tao, X.-Y. *et al.* (2007) Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nature Biotechnology* 25, 1307–1313.

Masucci, J. (2019) Lessons learned from developing an RNAi-based *Varroa* control product. Available at: www.apimondia.com (accessed 18 November 2021)

Mehlhorn, S., Ulrich, J., Baden, C.U., Buer, B., Maiwald, F. *et al.* (2021) The mustard leaf beetle, *Phaedon cochleariae*, as a screening model for exogenous RNAi-based control of coleopteran pests. *Pesticide Biochemistry and Physiology* 176, 104870.

Meng, L.W., Peng, M.L., Chen, M.L., Yuan, G.R., Zheng, L.S. *et al.* (2020) A glutathione S-transferase (BdGSTd9) participates in malathion resistance via directly depleting malathion and its toxic oxide malaoxon in *Bactrocera dorsalis* (Hendel). *Pest Management Science* 76, 2557–2568.

Mezzetti, B., Smagghe, G., Arpaia, S., Christieans, O., Dietz-Pfeilstetter, A. *et al.* (2020) RNAi: what is its position in agriculture? *Journal of Pest Science* 93, 1125–1130.

Miller, S.C., Miyata, K., Brown, S.J. and Tomoyasu, Y. (2012) Dissecting systemic RNA interference in the red flour beetle *Tribolium castaneum*: parameters affecting the efficiency of RNAi. *PLoS ONE* 7(10), e47431.

Mingels, L., Wynant, N., Santos, D., Peeters, P., Gansemans, Y. *et al.* (2020) Extracellular vesicles spread the RNA interference signal of *Tribolium castaneum* TcA cells. *Insect Biology and Molecular Biology* 122, 103377.

Mishra, S., Dee, J., Moar, W., Dufner-Beattie, J., Baum, J. *et al.* (2021) Selection for high levels of resistance to double-stranded RNA (dsRNA) in Colorado potato beetle (*Leptinotarsa decemlineata* Say) using non-transgenic foliar delivery. *Scientific Reports* 11, 1–12.

Mohammed, A.M.A. (2016) RNAi-based silencing of genes encoding the vacuolar ATPase subunits a and c in pink bollworm (*Pectinophora gossypiella*). *African Journal of Biotechnology* 15, 2547–2557.

Myles, K.M., Wiley, M.R., Morazzani, E.M. and Adelman, Z.N. (2008) Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America* 105, 19938–19943.

Niu, J., Shen, G., Christiaens, O., Smagghe, G., He, L. *et al.* (2018a) Beyond insects: current status, achievements and future perspectives of RNAi in mite pests. *Pest Management Science* 74, 2680–2687.

Niu, J.Z., Taning, C.N.T., Christiaens, O., Smagghe, G. and Wang, J.J. (2018b) Rethink RNAi in insect pest control: challenges and perspectives. *Advances in Insect Physiology* 55, 1–17.

Paldi, N., Glick, E., Oliva, M., Zilberberg, Y., Aubin, L. *et al.* (2010) Effective gene silencing in a microsporidian parasite associated with honeybee (*Apis mellifera*) colony declines. *Applied and Environmental Microbiology* 76, 5960–5964.

Pan, H., Yang, X., Bidne, K., Hellmich, R.L., Siegfried, B.D. *et al.* (2017) Dietary risk assessment of v-ATPase A dsRNA on monarch butterfly larvae. *Frontiers in Plant Science* 8, 242.

Park, Y., Kim, Y.J. and Adams, M.E. (2002) Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. *Proceedings of the National Academy of Sciences USA* 99, 11423–11428.

Petrick, J.S., Brower-Toland, B., Jackson, A.L. and Kier, L.D. (2013) Safety assessment of food and feed from biotechnology-derived crops employing RNA-mediated gene regulation to achieve desired traits: a scientific review. *Regulatory Toxicology Pharmacology* 66, 167–176.

Piot, N., Snoeck, S., Vanlede, M., Smagghe, G. and Meeus, I. (2015) The effect of oral administration of dsRNA on viral replication and mortality in *Bombus terrestris*. *Viruses* 7, 3172–3185.

Prentice, K., Pertry, I., Christiaens, O., Bauters, L., Bailey, A. *et al.* (2015) Transcriptome analysis and systemic RNAi response in the African sweetpotato weevil (*Cylas puncticollis, Coleoptera*, Brentidae). *PLoS ONE* 10, e0115336.

Prentice, K, Christiaens, O., Pertry, I., Bailey, A., Niblett, C. *et al.* (2017) RNAi-based gene silencing through dsRNA injection or ingestion against the African sweet potato weevil *Cylas puncticollis* (Coleoptera: Brentidae). *Pest Management Science* 73, 44–52.

Qiu, S., Adema, C.M. and Lane, T. (2005) A computational study of off-target effects of RNA interference. *Nucleic Acids Research* 33, 1834–1847.

Reid, W. and O'Brochta, D.A. (2016) Applications of genome editing in insects. *Current Opinion in Insect Science* 13, 43–54.

Riga, M., Denecke, S., Livadaras, I., Geibel, S., Nauen, R. *et al.* (2019) Development of efficient RNAi in *Nezara viridula* for use in insecticide target discovery. *Archives in Insect Biochemistry and Physiology* 1, 9.

Rodrigues, T.B., Rieske, L.K., Duan, J.J., Mogilicherla, K. and Palli, S.R. (2017) Development of RNAi method for screening candidate genes to control emerald ash borer, *Agrilus planipennis*. *Scientific Reports* 7, 7379.

Rodrigues, T.B., Duan, J.J., Palli, S.R. and Rieske, L.K. (2018) Identification of highly effective target genes for RNAi-mediated control of emerald ash borer, *Agrilus planipennis*. *Scientific Reports* 8, 5020.

Saleh, M.C., van Rij, R.P., Hekele, A., Gillis, A., Foley, E. *et al.* (2006) The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. *Nature Cell Biology* 8, 793–802.

San Miguel, K. and Scott, J.G. (2016) The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. *Pest Management Science* 72, 801–809.

Sarmah, N., Kaldis, A., Taning, C.N.T., Perdikis, D., Smagghe, G. *et al.* (2021) dsRNA-mediated pest management of *Tuta absoluta* is compatible with its biological control agent *Nesidiocoris tenuis*. *Insects* 12, 274.

Sato, K., Miyata, K., Ozawa, S. and Hasegawa, K. (2019) Systemic RNAi of V-ATPase subunit B causes molting defect and developmental abnormalities in *Periplaneta fuliginosa*. *Insect Science* 26, 721–731

Schnettler, E., Sterken, M.G., Leung, J.Y., Metz, S.W., Geertsema, C. *et al.* (2012) Noncoding flavivirus RNA displays RNA interference suppressor activity in insect and mammalian cells. *Journal of Virology* 86, 13486–13500.

Schoofs, L., De Loof, A. and Van Hiel, M.B. (2017) Neuropeptides as regulators of behavior in insects. *Annual Reviews of Entomology* 62, 35–52.

Schultheis, D., Weißkopf, M., Schaub, C., Ansari, S., Dao, V.A. *et al.* (2019) A large scale systemic RNAi screen in the red flour beetle *Tribolium castaneum* identifies novel genes involved in insect muscle development. *G3: Genes, Genomes, Genetics* 9, 1009–1026.

Scott, J.G., Michel, K., Bartholomay, L.C., Siegfried, B.D., Hunter, W.B. *et al.* (2013) Towards the elements of successful insect RNAi. *Journal of Insect Physiology* 59, 1212–1221.

Sharma, R., Taning, C.N.T., Smagghe, G. and Christiaens, O. (2021) Silencing of double-stranded ribonuclease improves oral RNAi efficacy in southern green stinkbug *Nezara viridula*. *Insects*, 12, 115.

Shi, Y., Jiang, H.-B., Gui, S.-H., Liu, X.-Q., Pei, Y.-X. *et al.* (2017) Ecdysis triggering hormone signaling (ETH/ETHR-A) is required for the larva-larva ecdysis in *Bactrocera dorsalis* (Diptera: Tephritidae). *Frontiers in Physiology* 8, 587.

Shi, Y., Liu, T.-Y., Jiang, H.-B., Liu, X.-Q., Dou, W. *et al.* (2019) The ecdysis triggering hormone system, via ETH/ETHR-B, is essential for successful reproduction of a major pest insect, *Bactrocera dorsalis* (Hendel). *Frontiers in Physiology* 10, 151.

Sigoillot, F.D., Lyman, S., Huckins, J.F., Adamson, B., Chung, E., *et al.* (2012) A bioinformatics method identifies prominent off-targeted transcripts in RNAi screens. *Nature methods* 9, 363–366.

Smagghe, G., Zotti, M. and Retnakaran, A. (2019) Targeting female reproduction in insects with biorational insecticides for pest management: a critical review with suggestions for future research. *Current Opinion in Insect Science* 31, 65–69.

Soberón, M., Pardo-López, L., López, I., Gómez, I., Tabashnik, B.E. *et al.* (2007) Engineering modified *Bt* toxins to counter insect resistance. *Science* 318, 1640–1642.

Sorek, R., Lawrence, C.M. and Wiedenheft, B. (2013) CRISPR-mediated adaptive immune systems in bacteria and archaea. *Annual Review of Biochemistry* 82, 237–266.

Taning, C.N.T., Van Eynde, B., Yu, N., Ma, S. and Smagghe, G. (2017) CRISPR/Cas9 in insects: applications, best practices and biosafety concerns. *Journal of Insect Physiology* 98, 245–257.

Taning, C.N.T., Christiaens, O., Li, X., Swevers, L., Casteels, H. *et al.* (2018). Engineered Flock House Virus for targeted gene suppression through RNAi in fruit flies (*Drosophila melanogaster*) in vitro and in vivo. *Frontiers in Physiology* 9, 805.

Taning, C.N.T., Arpaia, S., Christiaens, O., Dietz-Pfeilstetter, A., Jones, H. *et al.* (2019) RNA-based biocontrol compounds: current status and perspectives to reach the market. *Pest Management Science* 76, 841–845.

Taning, C.N.T., Mezzetti, B., Kleter, G., Smagghe, G. and Baraldi, E. (2020) Does RNAi-based technology fit within EU sustainability goals? *Trends in Biotechnology* 39, 644–647.

Taning, C.N.T., Gui, S., De Schutter, K., Jahani, M., Castellanos, N. *et al.* (2021) A sequence complementarity-based approach for evaluating off-target transcript knockdown in *Bombus terrestris*, following ingestion of pest-specific dsRNA. *Journal of Pest Science* 94, 487–503.

Terenius, O., Papanicolaou, A., Garbutt, J.S., Eleftherianos, I., Huvenne, H. *et al.* (2011) RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design. *Journal of Insect Physiology* 57, 231–245.

Tian, H., Peng, H., Yao, Q., Chen, H., Xie, Q. *et al.* (2009) Developmental control of a lepidopteran pest *Spodoptera exigua* by ingestion of bacteria expressing dsRNA of a non-midgut gene. *PLoS ONE* 4, e6225.

Tomoyasu, Y., Miller, S.C., Tomita, S., Schoppmeier, M., Grossmann, D. and Bucher, G. (2008) Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in *Tribolium*. *Genome Biology* 9, R10.

Tribolium Genome Sequencing Consortium (2008) The genome of the model beetle and pest *Tribolium castaneum*. *Nature* 452, 949–955.

Ulrich, J., Dao, V.A., Majumdar, U., Schmitt-Engel, C., Schwirz, J. *et al.* (2015) Large scale RNAi screen in *Tribolium* reveals novel target genes for pest control and the proteasome as prime target. *BMC Genomics* 16, 674.

vanEngelsdorp, D., Underwood, R., Caron, D. and Hayes, J. Jr (2007) An estimate of managed colony losses in the winter of 2006–2007: a report commissioned by the Apiary Inspectors of America. *American Bee Journal* 147, 599–603.

Vélez, A.M. and Fishilevich, E. (2018) The mysteries of insect RNAi: a focus on dsRNA uptake and transport. *Pesticide Biochemistry and Physiology* 151, 25–31.

Vélez, A.M., Fishilevich, E., Matz, N., Storer, N.P., Narva, K.E. *et al.* (2017) Parameters for successful parental RNAi as an insect pest management tool in western corn rootworm, *Diabrotica virgifera virgifera*. *Genes* 8, 1–18.

Vogel, E., Santos, D., Mingels, L., Verdonckt, T.-W. and Vanden Broeck, J. (2019) RNA Interference in insects: protecting beneficials and controlling pests. *Frontiers in Physiology* 9, 1912.

Walski, T., Van Damme, E.J.M., Smargiasso, N., Christiaens, O., De Pauw, E. *et al.* (2016) Protein N-glyco-sylation and N-glycan trimming are required for postembryonic development of the pest beetle *Tribolium castaneum*. *Scientific Reports* 6, 35151.

Wang, L.L., Lu, X.P., Meng, L.W., Huang, Y., Wei, D. *et al.* (2016) Functional characterization of an α-esterase gene involving malathion detoxification in *Bactrocera dorsalis* (Hendel). *Pesticide Biochemistry and Physiology* 130, 44–51.

Wang, N.M., Li, J.J., Shang, Z.Y., Yu, Q.T. and Xue, C.B. (2020) Increased responses of phenoloxidase in chlorantraniliprole resistance of *Plutella xylostella* (Lepidoptera: Plutellidae). *Journal of Insect Science* 20, 2.

Wei, D., Liu, Y.-W., Zhang, S.-U., Xu, H.-Q., Smagghe, G. *et al.* (2021) A male accessory gland specific gene *takeout2* regulates male mating success in *Bactrocera dorsalis* (Hendel). *Entomologia Generalis*, in press.

Werren, J.H., Loehlin, D.W. and Giebel, J.D. (2009) Larval RNAi in *Nasonia* (parasitoid wasp). *Cold Spring Harbor Protocols* 2009, pdb.prot5311.

Whitehead, K.A., Langer, R. and Anderson, D.G. (2009) Knocking down barriers: advances in siRNA delivery. *Nature Reviews in Drug Discovery* 8, 129–138.

Whyard, S., Singh, A.D. and Wong, S. (2009) Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochemistry and Molecular Biology* 39, 824–832.

Willow, J., Soonvald, L., Sulg, S., Kaasik, R., Silva, A.I. *et al.* (2020) First evidence of bud feeding-induced RNAi in a crop pest via exogenous application of dsRNA. *Insects* 11, 769.

Willow, J., Soonvald, L., Sulg, S., Kaasik, R., Silva, A.I. *et al.* (2021) RNAi efficacy is enhanced by chronic dsRNA feeding in pollen beetle. *Communications Biology* 4, 444.

Winston, W.M., Molodowitch, C. and Hunter, C.P. (2002) Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. *Science* 295, 2456–2459.

Wu, Q., Wang, X. and Ding, S.-W. (2010) Viral suppressors of RNA-based viral immunity: host targets. *Cell Host & Microbe* 8, 12–15.

Wuriyanghan, H. and Falk, B.W. (2013) RNA interference towards the potato psyllid, *Bactericera cockerelli*, is induced in plants infected with recombinant Tobacco mosaic virus (TMV). *PLoS ONE* 8, e66050.

Xu, J., Xu, X., Zhan, S. and Huang, Y. (2019) Genome editing in insects: current status and challenges. *National Science Review* 6, 399–401.

Xu, L., Jiang, H.-B., Chen, X.-F., Xiong, Y., Lu, X.-P. *et al.* (2018) How tyramine-hydroxylase controls the production of octopamine, modulating the mobility of beetles. *International Journal of Molecular Sciences* 19, 846.

Yang, Q., De Schutter, K., Chen, P., Van Damme, E.J.M. and Smagghe, G. (2021) RNAi of the N-glycosylation-related genes confirms their importance in insect development and α-1,6-fucosyltransferase plays a role in the ecdysis event for the hemimetabolous pest insect *Nilaparvata lugens*. *Insect Science* 29(1), 91–99.

Yao, J., Rotenberg, D., Afsharifar, A., Barandoc-Alviar, K. and Whitfield, A.E. (2013) Development of RNAi methods for *Peregrinus maidis*, the corn planthopper. *PLoS ONE* 8, e70243.

Ye, C., Wang, Z.-W., Sheng, Y.-L., Wang, Z.-G., Smagghe, G. *et al.* (2021) *GNBP1* as a potential RNAi target to enhance the virulence of *Beauveria bassiana* for aphid control. *Journal of Pest Science* 95(1), 87–100.

Zhu, F., Parthasarathy, R., Bai, H., Woithe, K., Kaussmann, M. *et al.* (2010) A brain-specific cytochrome P450 responsible for the majority of deltamethrin resistance in the QTC279 strain of *Tribolium castaneum*. *Proceedings of the National Academy of Sciences of the USA* 107, 8557–8562.

Zhu, K.Y. and Palli, S.R. (2020) Mechanisms, applications, and challenges of insect RNA interference. *Annual Review of Entomology* 65, 293–311.

Zotti, M.J. and Smagghe, G. (2015) RNAi technology for insect management and protection of beneficial insects from diseases: Lessons, challenges and risk assessments. *Neotropical Entomology* 44, 197–213.

Zotti, M., Dos Santos, E.A., Cagliari, D., Christiaens, O., Taning, C.N.T. *et al.* (2018) RNA interference technology in crop protection against arthropod pests, pathogens and nematodes. *Pest Management Science* 74, 1239–1250.

5 Site-Specific Recombination for Gene Locus-Directed Transgene Integration and Modification

Hassan M. M. Ahmed and Ernst A. Wimmer*

Dept of Developmental Biology, Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology, GZMB, Georg-August-University Göttingen, Göttingen, Germany

5.1 Introduction

The ability to manipulate or engineer genomes of living organisms by introduction of new genetic information has revolutionized basic and applied research alike and opened new doors for functional genetic analysis and applied biotechnology. In insects, this was initially made possible by the discovery and use of transposable elements as gene vectors. The first successful deliberate germline transformation was the manipulation of *Drosophila melanogaster* using the *P* element (Rubin and Spradling, 1982; Spradling and Rubin, 1982). This has paved the way for an unprecedented number of discoveries and become instrumental in early functional and developmental genetics studies in *Drosophila*: insertional mutagenesis screens have provided loss-of-function (LOF) alleles of genes, whereas expression systems led to gain-of-function (GOF) situations. Unfortunately, the use of *P* element-based vectors for insects outside of the *Drosophila* group was not successful, due to the requirement for a host-specific factor necessary for transposition (O'Brochta and Handler, 1988; Rio and Rubin, 1988). Other vectors based on transposable elements

such as *mariner*, *Minos*, *Hermes* and importantly *piggyBac* were developed and used to engineer different insect taxa (Skipper *et al.*, 2013) (see O'Brochta, Chapter 1, this volume). The *piggyBac* transposon has been widely used to manipulate not only insects but also other animals, including mammals, and can therefore be considered a universal vector (Ding *et al.*, 2005).

Despite the fact that transposons remain an important tool for germline transformation in insects, transposon-based vectors have the inherent drawback that they integrate randomly in the genome and, as a result, the inserted transgene is exposed to position effects. This means that the surrounding genomic environment such as enhancer elements, silencers, or the state of local chromatin configuration can affect the activity of the transgene (Spradling and Rubin, 1983; Henikoff, 1992). This makes it difficult to compare diverse transgene activities at different genomic positions. To overcome this obstacle, scientists have engineered and exploited different strategies based on genetic recombination to introduce transgenes to edit the genome site-specifically or sequence-specifically.

---

*Corresponding author, email: ewimmer@gwdg.de

5.2   Classification and Mechanisms of Site-Specific Recombination

Broadly speaking, genetic recombination refers to the process of exchange of genetic information within or between DNA molecules (Carroll, 2013). In sexually reproducing eukaryotes, recombination takes place between homologous chromosomes during meiosis in the process of gamete formation (Thacker and Keeney, 2016). Homologous recombination is also involved in the repair process of DNA double-strand breaks (DSBs) (Moynahan and Jasin, 2010). Therefore, the introduction of DSBs can be used to cause recombination-mediated genome modifications. Besides, site-specific recombination (SSR) (Craig, 1988; Parks and Peters, 2018) is utilized mainly by temperate viruses such as bacteriophages, transposable elements, or plasmids in prokaryotes or single-cell eukaryotes, to get their genetic material integrated and multiplied along with the host genome or as a mechanism to ensure high copy numbers of the plasmid sequences (Grindley *et al*., 2006). The process is mediated by enzymes known as site-specific recombinases which bind to short recognition sequences (typically 30–200 bp) and facilitate recombination between two DNA molecules. Based on an amino acid residue in the active site involved in the nucleophilic attack of the DNA backbone, recombinases are classified into two structurally and mechanistically distinct families: tyrosine or serine site-specific recombinases.

5.2.1   Tyrosine and serine site-specific recombinases

The founding and most studied member of the tyrosine site-specific recombinase (T-SSR) family is lambda integrase (λ int), which is encoded in the genome of and used by the temperate virus *Escherichia phage lambda* during its lysogenic cycle to mediate integration and excision of its genome from a specific site on the chromosome of *Escherichia coli* (Campbell, 1963; Landy, 1989). Despite being the first to be described, λ int was not used in genome modification, due to the requirement for a host-encoded integration factor (Nash and Robertson, 1981; Friedman, 1992), which made its use in heterologous systems not feasible. The most common tyrosine site-specific recombinases used in heterologous situations are the Cre-*Lox* (causes recombination, locus of crossover (x) (of P1)) system, isolated some 40 years ago from the *P1* bacteriophage (Sternberg and Hamilton, 1981), and the Flp-*FRT* (flippase, Flp recombination target) system from the 2µ plasmid of the baker's yeast *Saccharomyces cerevisae* (Broach *et al*., 1982). The two tyrosine recombinases require no accessory factors to catalyse the recombination reaction and therefore have been adopted as tools for genome modification in diverse model organisms. Both Cre and Flp catalyse reversible recombination reactions between two identical recognition sequences: *LoxP* for Cre and *FRT* for Flp (Venken and Bellen, 2005). The *LoxP* site is 34 bp with a 13 bp palindromic sequence, which Cre binds, and an 8 bp intervening spacer, where crossover takes place. Engineering hetero-specific *Lox* sites by changing a few nucleotides in the 8 bp spacer increased the applicability of the system as a tool for transgenesis. Interestingly, Flp also recognizes a 34 bp *FRT* site with an 8 bp spacer. To increase its applications, the spacer has also been mutated to generate a set of hetero-specific *FRT* sites (Bode *et al*., 2005) (see 'φC31 integrase mediated single site integration' in section 5.3.1, below).

However, the integration of a transgene into a single recognition site using the Cre-*Lox* or Flp-*FRT* systems is hardly possible, because the respective enzymes induce recombination between two identical recognition sequences. Therefore, both the forward (integration) and the reverse (excision) reactions are mediated by the same enzyme. Since the *cis* recombination (both sites on the chromosome) is kinetically more favourable than the *trans* recombination (one site in chromosome and one site on plasmid), the net outcome is excision rather than integration of the transgene (Fig. 5.1A) (Baer and Bode, 2001). Nevertheless, based on the position and
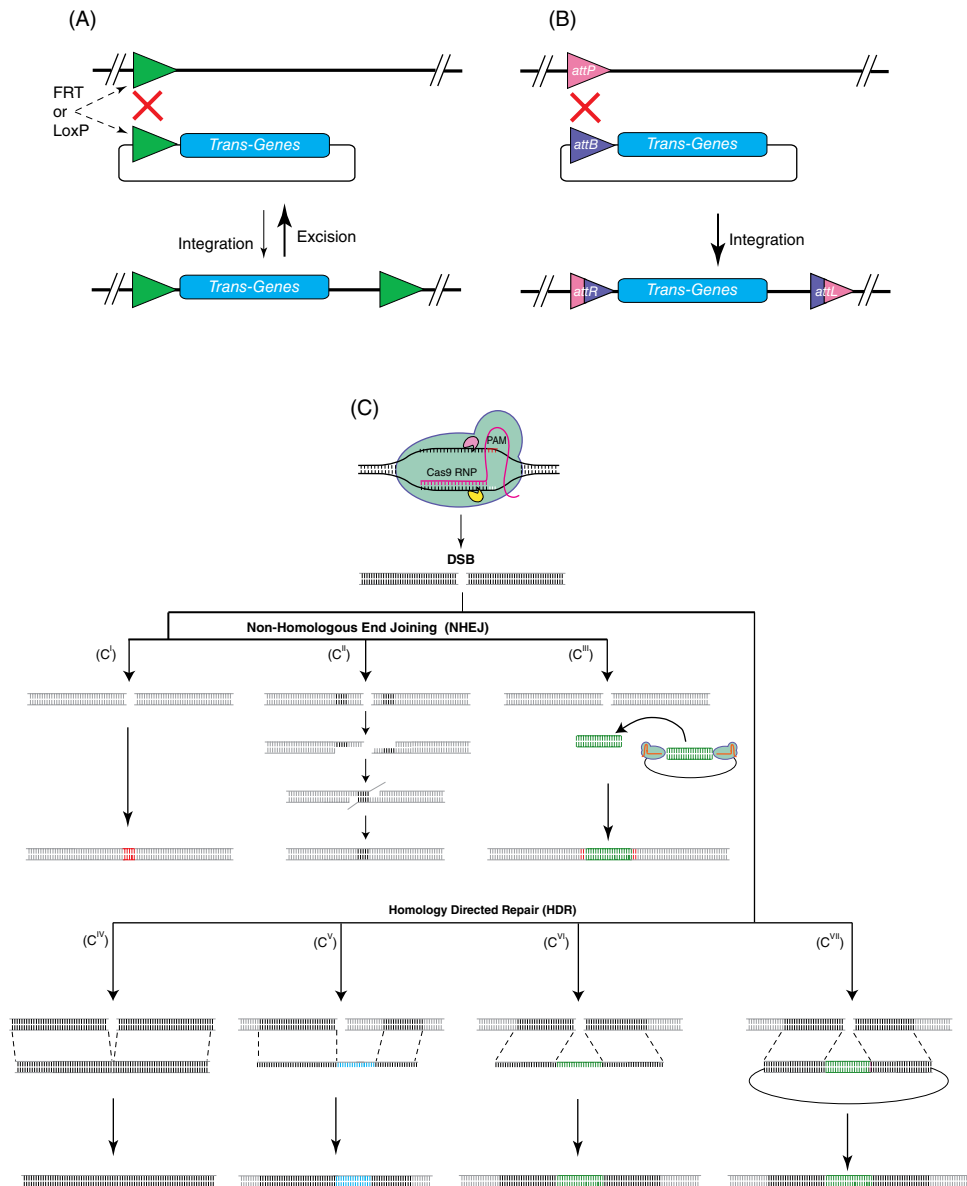
Fig. 5.1. Transgenesis by site-specific recombination. The red cross indicates recombination between recombinase target sequences in the genome and the donor plasmid. **(A)** In the case of the FLP-*FRT* and Cre-*Lox* SSR systems, the excision reaction between two identical recombinase recognition sequences (RRSs) (green triangle) is more favourable over the integration, which is indicated by the thick arrow (reviewed in Wimmer, 2005). **(B)** The φC31 integrase mediates recombination between two heterologous RRSs, *attP* (pink triangle) and *attB* (purple triangle), and results in integration of the whole plasmid carrying the transgenes (blue box) as well as antibiotic resistance (black line), creating two different hybrid sites, *attR* and *attL* (mixed pink/purple triangles) (Thorpe *et al*., 2000). **(C)** CRISPR/Cas9-induced double-strand breaks (DSBs) can be repaired by error-prone (indicated in red) mechanisms such as lig IV-dependent non-homologous end joining (NHEJ) (C^I) or ligase III-dependent microhomology-mediated

orientation of two identical copies of the recognition sequence, genome modifications such as deletion, inversion and translocations can be performed, respectively (see 'Chromosome engineering by site-specific recombination' in section 5.3.4, below).

In contrast, members of the serine site-specific recombinase (S-SSR) family are known to catalyse a unidirectional recombination between two different recognition sites called attachment sites (*att*), which can be reversed in the presence of a protein called recombination directionality factor (RDF) (Rutherford *et al.*, 2013). This fact made S-SSR one of the best choices for genetic engineering in heterologous systems. The most commonly used S-SSR is φC31 integrase derived from the *Streptomyces* phage φC31 (Rausch and Lehmann, 1991), which mediates the integration of the phage genome by recombination between two non-identical *att* sites: the phage attachment site (*attP*) and the bacterial attachment site (*attB*). The recombination then leads to the integration of the phage genome into the bacterial chromosome and the generation of two hybrid sites called *attL* and *attR* (Fig. 5.1B) (Thorpe *et al.*, 2000). In heterologous systems this integration reaction is unidirectional, because the resultant *attR* and *attL* sites are no longer substrate for the integrase enzyme alone and therefore the net outcome is highly efficient integration. This is the main advantage of φC31 integrase over the tyrosine recombinases systems Cre-*Lox* and Flp-*FRT*.

5.2.2 CRISPR/Cas-mediated DNA double-strand breaks for site-specific genome editing

In addition to the classical recombinases mentioned above, site-specific genome targeting was attempted using homologous recombination, albeit with very low efficiency (Aizawa, 1995; Rong and Golic, 2000; Gong and Golic, 2003). However, it has been found that induction of DSBs increases the efficiency of homologous recombination by at least two orders of magnitude (Rouet *et al.*, 1994), which prompted the development and use of natural and synthetic endonucleases for this purpose. DSBs can be induced by means of rare cutting homing endonucleases such as I-*SceI* (Jasin, 1996; Rouet *et al.*, 1994), designer nucleases such as zinc-finger nucleases (ZFNs) (Urnov *et al.*, 2010) or transcription activator-like endonuclease (TALENs) (Miller *et al.*, 2011), and by the latest technology of genome editing: the clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein (Cas) system (Jinek *et al.*, 2012; Cong *et al.*, 2013). The latter is an adaptive immune system used by bacteria and archaea to fight against parasitic DNA elements such as bacteriophages and transposable elements (Makarova *et al.*, 2006; Barrangou *et al.*, 2007; Brouns *et al.*, 2008) (see Concha and Papa, Chapter 7, this volume). The system consists of a Cas endonuclease (Cas9) and two RNAs: the CRISPR-RNA (crRNA) and the *trans*-activating CRISPR RNA (tracrRNA). The crRNA

Fig. 5.1. Continued.

end joining (MMEJ) (C[II]) (reviewed in McVey and Lee, 2008) using microhomology regions (indicated in black). The NHEJ pathway can also be exploited to knock-in large linear double-stranded DNA fragments (indicated in green) into the genome (C[III]) (Auer *et al.*, 2014). The strategy involves co-injection along with Cas9 and the target-specific gRNA of an extra plasmid containing the DNA to be integrated, flanked by Cas9 target sites to facilitate linearization of the fragment (He *et al.*, 2019). DSBs can also be repaired by a more precise mechanism, homology-directed repair (HDR), that naturally relies on the presence of homologous (indicated in black) chromosomes (C[IV]) (Liang *et al.*, 1998; Bibikova *et al.*, 2002). HDR has been used in genome editing to introduce site-specific genome modifications such as a change of one or a few nucleotides (indicated in blue) using a single-strand oligonucleotide with short homology regions (indicated in black) as repair template (C[V]) (Gratz *et al.*, 2013). However, the pathway has also been used to knock-in transgenes of interest (indicated in green) using single- (C[VI]) or double-stranded DNA (C[VII]) as repair templates containing left and right homology arms (indicated in black) (Port *et al.*, 2014; Kanca *et al.*, 2019).

is 42 nt long, contains about 20 nt specific to the genomic target, and guides the Cas9 to the specific genomic target by complementary base-pairing (Gasiunas *et al*., 2012). tracrRNA is involved along with Cas9 in processing and maturation of the crRNA and also mediates the interaction between crRNA and Cas9 (Deltcheva *et al*., 2011). For ease of use or *in vitro* transcription of the two RNAs, scientists have engineered them into a single chimeric guide RNA (*gRNA*) (Bassett *et al*., 2013). CRISPR/Cas can in theory be easily programmed to target any 17–20 bp genomic sequence to induce DSBs for generating desired modifications (Fig. 5.1C). The only prerequisite is the availability of a PAM (protospacer adjacent motif), which in the case of the most commonly used Cas9 from *Streptococcus pyogenes* (SpCas9) is the sequence *NGG*, where *N* stands for any nucleotide. As well as genome editing, several CRISPR/Cas systems have been discovered and tailored to perform different tasks such as gene activation and repression, base editing and CRISPR screens, among others, which are beyond the scope of this chapter. Here we focus on the use of CRISPR-Cas9 in insects for site-specific genome modifications.

5.3 Applications of Site-Specific Recombination

5.3.1 Integration into a single specific site

CRISPR/Cas 9 genome editing

The CRISPR/Cas9 system was first used in mammalian cell culture (Cong *et al*., 2013; Mali *et al*., 2013), mouse (Platt *et al*., 2014) and zebra fish (Hwang *et al*., 2013), which demonstrated its feasibility to induce site-specific genome modifications. Following the first demonstration that CRISPR/Cas9 could induce heritable site-specific genome modification in the model organism *D. melanogaster* (Gratz *et al*., 2013), researchers have adopted and established the system in many more insect species, including insects of agricultural and veterinary importance and vectors of human diseases (Reid and O'Brochta, 2016).

The system was used in basic research in functional genetic analyses as well as in applied biotechnology to develop novel transgenic pest control strategies based on gene drive (see Champer, Chapter 9, this volume). The role of CRISPR/Cas9 in genome modification is limited to the generation of DSBs. The rest is taken care of by the cell machineries of DSB repair.

The main pathways involved in the repair of DSBs depicted in Fig. 5.1C are non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Liang *et al*., 1998; Bibikova *et al*., 2002). The former acts fast to repair DSBs by an error-prone joining and ligating of the two ends of the broken DNA molecule often resulting in insertion or deletion of a few nucleotides, which may lead to gene knockout by frame-shift and premature stop codons (Fig. 5.1C[I]) (Bibikova *et al*., 2002). In contrast, HDR is more precise but is limited to situations of the close presence of a homologous DNA molecule to repair a DSB by homologous recombination (Fig. 5.1C[IV]). A third and distinct DSB repair pathway is the alternative non-homologous end joining also referred to as microhomology-mediated end joining (MMEJ) (Fig. 5.1C[II]). This pathway relies on annealing of minimal homology between the cut strands from 1 to 14 nt usually causing small deletions. MMEJ depends on ligase III to join the two ends together, whereas the classical NHEJ uses ligase IV (Nussenzweig and Nussenzweig, 2007; McVey and Lee, 2008).

The two main pathways involved in the repair of DSBs have been intensively exploited to introduce different site-specific genome modifications, including gene knockout and knock-in. Importantly, NHEJ can be used to integrate large transgenes (Ishibashi *et al*., 2006). In this case, the transgene to be integrated has to be available as linear dsDNA molecule at the time of repair of the DSB (Auer *et al*., 2014; Vaidya *et al*., 2014). The enzymes involved in this pathway will indiscriminately use DNA ends as substrate and join them together (Ishibashi *et al*., 2006). The dsDNA to be incorporated in the genome by NHEJ can be delivered as linear PCR product or as a plasmid vector that can be linearized *in vivo* using a site-specific endonuclease (Fig. 5.1C[III]) (Auer *et al*., 2014). The

NHEJ pathway had already been exploited as a strategy for site-specific integration of transgenes by direct ligation of an *in vivo* linearized plasmid into a DSB generated by ZFN which they named ObLiGaRe, an abbreviation for <u>Ob</u>ligate <u>Li</u>gation-<u>Ga</u>ted <u>Re</u>combination (Maresca *et al.*, 2013). With this strategy, they were able to integrate a 15 kb fragment into the genome of human cell lines at a defined locus. Studies also demonstrated that co-injection of a circular plasmid that will be linearized *in vivo* concurrently with the attack of the genome target is less toxic and more efficient (Cristea *et al.*, 2013; Auer *et al.*, 2014). In their work to study the function of the transcription factor Tc-foxQ2 in brain development of the red flour beetle *Tribolium castaneum*, He *et al.* (2019) exploited the CRISPR/Cas9 NHEJ-knock-in system to generate a targeted enhancer trap situation, in which the donor template was concomitantly linearized *in vivo* at a single target site by Cas9. This strategy has the disadvantage of integration of the whole vector, including the antibiotic resistance gene. Inclusion of such huge unnecessary DNA can be omitted by introduction of two endonuclease sites flanking the transgene to be inserted (Fig. 5.1C[III]) or by inclusion of *FRT* or *Lox* sites to facilitate removal of the backbone by site-specific recombination-mediated excision in a separate later step to modify the transgene insertion (see also 'Modification and stabilization of transgenes' in section 5.3.3, below).

In contrast, site-directed integration by HDR is less efficient but more precise than NHEJ. In general, the efficiency of HDR increases with the length of the homology arms of the repair template (Fig. 5.1C[V]–C[VII]) but drops with an increase of the cargo size over 2 kb (Li *et al.*, 2014; Wang *et al.*, 2015). Therefore, this strategy is more suitable for small modifications causing directed precise genome editing or integration of short transgenic sequences. As repair templates, single-strand oligodeoxynucleotides (ssODN) (Kanca *et al.*, 2019; Aumann *et al.*, 2020) or a circular double-strand donor can be used (Fig. 5.1C[V]–C[VII]). Linear double-strand donor can be integrated by NHEJ and is therefore usually not used in HDR. Replacement of the *yellow* gene of *D. melanogaster* with an

attP site was the first demonstration of HDR using short ssODN as repair template (Gratz *et al.*, 2013). *In vivo* gene tagging is another useful application for HDR, such as fusion of the coding sequence of the *green fluorescent protein* (*GFP*) (Shimomura *et al.*, 1962) mostly at the N- or C-terminal end in-frame with the protein to be studied. In their early attempts to establish Cas9 as a genome modification tool for insects, Port *et al.* (2014) were able to insert the coding sequence of *GFP* in-frame in the *Drosophila wingless* (*wg*) gene and showed that *GFP* followed the same pattern of *wg* expression and that it was secreted as well. Instead of generating a fusion protein, a bicistronic situation can also be achieved by using the viral 2A peptide strategy to mark certain cells (Farnworth *et al.*, 2020a,b).

Strategies that increase the overall efficiency of generating Cas9-induced DSBs have also been shown to increase the chance of integration of transgenes by HDR. These include the form of delivery of the different components. For example, Cas9 can be delivered as ribonucleoprotein complex ready to cut the target, as mRNA, or as a helper plasmid with Cas9 coding sequence under different *cis*-regulatory elements such as *heat shock protein* promoters. However, generation of transgenic lines that express Cas9 from promoters of germline-specific or ubiquitous genes such as *vasa* and *nanos* or *Actin 5*, respectively, was found to be the most efficient and cost-effective alternative to all other strategies (Port *et al.*, 2014; Kandul *et al.*, 2021; Yan *et al.*, 2021). The second component of the system is the specific gRNA, which guides Cas9 to where to cut. The 17–20 nt at the 5′ end of the gRNAs need to be designed for each specific genomic target. The gRNA can be delivered in the form of synthetic or *in vitro* transcribed RNA and this form is compatible with Cas9 being delivered as mRNA or protein. It can also be delivered in the form of plasmid to be expressed from promoters of *RNA pol III* genes such as the *small nuclear RNA gene U6*; however, not all promoters of *U6* genes of the same organism are equally strong. In *D. melanogaster*, there are three copies of the *U6* gene and the promoter of the *U6:3* gene was found to be the strongest (Port *et al.*, 2014).

The same was observed in *Drosophila suzukii*, in which the equivalent of the *U6:3* promoter referred to as *U6c* outperformed the other two in the overall efficiency of HDR-based knock-in (Ahmed *et al.*, 2019).

NHEJ competes with HDR to resolve DSBs. To favour HDR, several strategies have been followed. The use of tight germline-specific promotors seems to increase the rate of HDR in homing-based gene drive applications of CRISPR/Cas9 (Champer *et al.*, 2018). In addition, knockdown or knockout of genes involved in the NHEJ pathway were proposed as strategies to improve on the efficiency of integration by HDR. Attempts to silence or use strains with null mutations of the *ligase 4* (*lig4*) gene – the enzyme involved in NHEJ repair – showed dramatic improvement in HDR in *D. melanogaster* (Beumer *et al.*, 2008). Knockout of *Ku70* was found to increase HDR in the silkworm *Bombyx mori* embryos (Ma *et al.*, 2014). Highly efficient HDR was also observed in *B. mori* ovarian cells that lack proteins important for NHEJ such as lig4, Ku70, Ku80 and XRCC4 (Zhu *et al.*, 2015). In the yellow fever mosquito *Aedes aegypti*, knockdown of *Ku70* and *lig4* increased HDR-based knock-in from 0.1% to more than 2% (Basu *et al.*, 2015).

The cargo size limitation of integration by HDR has been solved in many instances by a two-step strategy, where Cas9 is used to site-specifically introduce first by HDR recombinase recognition sequences (RRSs) such as *attP* (Gratz *et al.*, 2013; Carrami *et al.*, 2018). In a second step, the transgene will then be introduced by SSR (see 'Recombinase-mediated cassette exchange (RMCE)' in section 5.3.2, below). Cas9-mediated integration is a very useful tool when specific genes are targeted or suitable genomic loci have been identified that support expression of the transgene of interest. However, for many emerging model systems or non-model organisms such as agricultural pests and disease vectors, there is only limited information on suitable genomic target sites for successful expression of transgenic constructs. In these cases, SSR target sites for integration (see 'ϕC31 integrase mediated single site integration', below) or recombination-mediated cassette exchange (RMCE) (see 'Recombinase-mediated cassette exchange (RMCE)' in section

5.3.2, below) can be introduced by transposon-mediated random genome insertion (Eckermann *et al.*, 2018). To identify the most usable sites, they need to be characterized and evaluated for their suitability to express transgenes stably at sufficient levels and with minimal position effects.

ϕC31 integrase mediated single-site integration

Before the discovery or engineering of programmable endonucleases such as ZFN, TALENs and CRISPR/Cas9, SSR was routinely used for site-specific genome modification. For single-site integration into insect genomes, the *attP/attB* ϕC31 integrase system is most commonly used. In a heterologous situation, usually the phage-derived *attP* site is stably integrated into the genome of the target organism where it serves as a landing or docking site for a donor plasmid harbouring the transgene of interest and the bacterial *attB* sequence (Fig. 5.1B) (Belteki *et al.*, 2003). The major advantage of this system as a tool for genetic engineering is that the reaction is directional and the transgene, once integrated, cannot be excised by ϕC31 integrase alone. The results of recombination between *attP* and *attB* are the hybrid sites *attL* and *attR*, which require for the reverse excision reaction, in addition to the integrase, the phage encoded RDF gp3 (Fogg *et al.*, 2018). This system has been successfully established to engineer several model and non-model insects, including *D. melanogaster* (Groth *et al.*, 2004), the medfly *Ceratitis capitata* (Schetelig *et al.*, 2009), the malaria vectors *Anopheles gambiae* and *Anopheles stephensi* (Amenya *et al.*, 2010; Meredith *et al.*, 2011), *Ae. aegypti* and *Aedes albopictus* (Nimmo *et al.*, 2006; Labbé *et al.*, 2010; Franz *et al.*, 2011), as well as the cherry vinegar fly *D. suzukii* (Ahmed *et al.*, 2020). The efficiency of ϕC31 integrase was improved by fusion of a nuclear localization signal at the C-terminal of the enzyme (Andreas *et al.*, 2002). In *D. melanogaster*, the use of a transgenic source of ϕC31 integrase using a germline-specific promoter achieved more than 60% efficiency of integration (Bischof *et al.*, 2007). An additional advantage of ϕC31 integrase is the

unlimited cargo size, which has been demonstrated by integration of a complete 133 kb bacterial artificial chromosome into the genome of *D. melanogaster* (Venken *et al.*, 2006). However, the main disadvantage of a single-site *attP/attB* SSR is that the whole donor vector is integrated, which also includes the antibiotic resistance gene. This is of particular concern when it comes to transgene-based pest control strategies with the final aim to release the transgenic insects in the field. This issue can be addressed by including additional independent SSR sites in the donor plasmid that allow the subsequent deletion of unwanted components (Schetelig *et al.*, 2009) (see 'Modification and stabilization of transgenes' in section 5.3.3, below).

5.3.2 Integration into two sites

Recombinase-mediated cassette exchange (RMCE)

Since the use of the Cre-*Lox* or Flp-*FRT* systems for integration at a single site is not possible (Fig. 5.1A), a more sophisticated strategy, termed RMCE, was developed making use of mutant versions of the *FRT* and *Lox* sites (Fig. 5.2A–C) (Bouhassira *et al.*, 1997; Baer and Bode, 2001). Similar to the integration at a single recombination recognition sequence mentioned above, this strategy is also composed of two steps. In a first step, docking or landing lines are generated by introduction of a transformation marker flanked by two different *FRT* (e.g. *FRT* and *FRT3*) or *Lox* (e.g. *LoxP* and *Lox2272*) sites in a head-to-tail orientation in the genome of the organism of interest. In a second step, a donor vector carrying the transgene of interest flanked by the same two different *FRT* or *Lox* sites in a head-to-tail arrangement is delivered along with the respective recombinase enzymes flippase or Cre, respectively. A double reciprocal recombination between identical RRSs leads to the exchange of the genomically integrated cassette between the RRSs for the identically flanked cassette of the donor vector (Fig. 5.2A). RMCE based on the Flp-*FRT* system has been used more often in insects than the Cre-*Lox* system.

The Flp-*FRT* system has been used in *D. melanogaster* with an efficiency of 23% (Horn and Handler, 2005), as well as in the silkworm *B. mori* (Long *et al.*, 2012) and an Sf9 insect cell line (Fernandes *et al.*, 2012). The Cre-*Lox* system has been used recently in *D. melanogaster* (Oberstein *et al.*, 2005), *Ae. aegypti* (Huang *et al.*, 2009; Häcker *et al.*, 2017) and *D. suzukii* (Schetelig *et al.*, 2019).

In addition, to develop RMCE strategies based on φC31 integrase, a transformation marker flanked by *attP* sites in a head-to-head arrangement has been introduced in the genome of the organism which will serve as a landing or docking site for future transgenesis applications. To perform RMCE, a donor vector with the transgene of interest flanked by bacterial *attB* sites in a head-to-head arrangement is delivered along with the φC31 integrase, which results in double reciprocal recombination between the *attP*s and *attB*s, leading to cassette exchange (Fig. 5.2B). The integration of the transgene is not directional. Thus, the orientation of the integrated cassette needs to be identified molecularly after integration. RMCE based on φC31 integrase has gained more popularity among insect scientists and has been established in *D. melanogaster* (Bateman *et al.*, 2006), *Ae. aegypti*, *Plutella xylostella* (Haghighat-Khah *et al.*, 2015) and *B. mori* (Long *et al.*, 2015). The RMCE system based on φC31 integrase has further been improved by introducing in the first step, in addition to a transformation marker, a transgene that expresses the φC31 integrase from an inducible or germline-specific promoter into the cassette flanked by the *attP* sites (Fig. 5.2C). Such docking lines are referred to as 'self-docking', since they provide the enzyme required for the recombination reaction and preclude the need to provide the enzyme as mRNA or plasmid, which can improve the efficiency of transgenesis. This approach has been established in *An. gambiae* (Meredith *et al.*, 2013; Pondeville *et al.*, 2014).

Double-strand break-induced recombination-mediated exchange (RME)

The clearest advantage of the RMCE systems is that only the transgene of interest is introduced
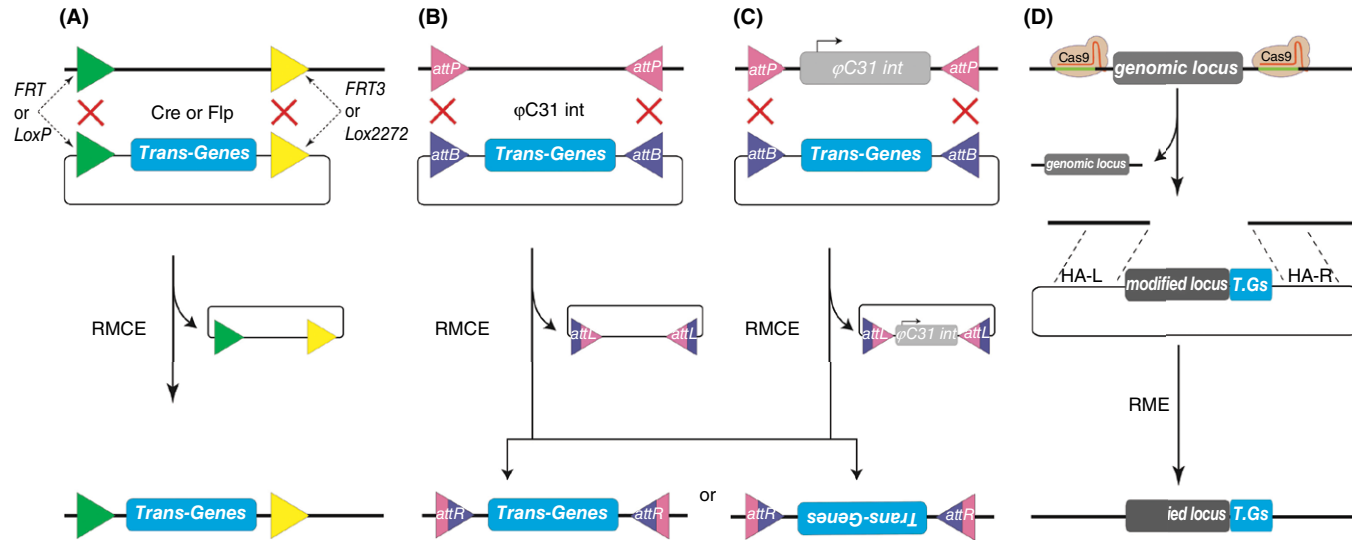
Fig. 5.2. Transgenesis by recombination mediated exchange. (A) Recombinase-mediated cassette exchange (RMCE) (Baer and Bode, 2001) takes place by recombination of two identical *FRT* or *Lox* sites and variations of these sites flanking a transgene of interest in the integrated transgene construct as well as in the donor plasmid, leading to exchange of the old transgene with the new transgene. **(B)** φC31 integrase-mediated cassette exchange of an integrated transgene flanked by two *attP* sites arranged head-to-head with a new transgene flanked by two *attB* sites in the same arrangement in the donor plasmid (Belteki *et al*., 2003). **(C)** The efficiency of the φC31 integrase-based RMCE can be improved by transgenic expression of the φC31 integrase (Meredith *et al*., 2013; Pondeville *et al*., 2014). The advantage of RMCE over integration in a single site is that the backbone is not integrated in the genome. **(D)** CRISPR/Cas9 can be used to exchange a defective gene with a wild-type version and vice versa. In this case, two DSBs flanking the locus to be exchanged are introduced and a repair template with homology arms (HA) is provided to achieve recombination-mediated exchange (RME) (Li and Handler, 2017) by which also additional transgenes (TGs) can be introduced.

and not the whole plasmid vector. More recently, CRISPR/Cas9 has also offered a strategy for targeted gene locus-specific recombination mediated exchange (RME) Fig. 5.2D). In this strategy, two DSBs flanking the locus of interest are introduced by Cas9, and a repair template is provided to replace the locus by reintroducing the locus with the desired modification. In this way the *yellow* locus in *D. melanogaster* was replaced by an *attP* site (Gratz *et al.*, 2013). To generate a temperature-sensitive allele in *D. suzukii*, a modified *transformer-2* locus along with a transformation marker was used to exchange the wild-type locus (Li and Handler, 2017).

Site-specific integration and RMCE/RME represent a major advantage, as they will allow more detailed comparative analyses of transgenes, eliminating variable genomic position effects. Moreover, transgenesis by SSR will have a major impact on non-model insects. After 'landing sites' have been generated by transposon-mediated random transgenesis or CRISPR/Cas9 genome editing, suitable loci can be identified in a given insect species. Since evaluated lines can be reused for new modifications, fewer lines need to be generated, examined and kept. For biotechnologically improved approaches to fight insect pests by a transgenic sterile insect technique (SIT), identification of genomic loci at which integration does not cause any major fitness cost will be of high interest, since transgenes could be integrated again and again at these particularly well-suited genomic target sites (Wimmer, 2005; Franz *et al.*, 2011).

5.3.3   Modification of transgenes

Mosaic transgene activiation

Ectopic gene expression to over- or misexpressed genes (Brand *et al.*, 1994; Halder *et al.*, 1995) or to knockdown genes by induced sequence-specific RNA interference (Schmitt-Engel *et al.*, 2015; Heigwer *et al.*, 2018) have been extensively used in functional genetic analysis causing GOF or LOF situations, respectively. These have been achieved by transgenic expression of the gene of interest

by gene fusion to a tissue- or stage-specific enhancer/promoter or a heat-shock inducible promoter upstream of the coding sequence of the gene of interest or to an inverted sequence generating double-stranded RNA (dsRNA) (Lee and Carthew, 2003). In many instances, constitutive ectopic expression is not suitable for gene analysis, especially when the gene in question is involved in different developmental programmes, and its mutant or overexpression leads to either sterility or lethality. In such cases, conditional tissue- or stage-specific GOF or LOF is advantageous. Different strategies based on the use of binary expression systems (see Schetelig *et al.*, Chapter 2, this volume) and/or recombination technologies such as the Cre-*Lox* or Flp-*FRT* systems were developed to achieve limited activation of the transgene, which enables studying the function of the gene in a particular tissue or at a specific stage.

In *D. melanogaster*, a smart strategy to control ectopic gene expression was developed by use of the Flp-*FRT* SSR system (Struhl and Basler, 1993). A constitutive or tissue-specific promoter is separated from the gene of interest by a *flip-out* cassette which contains a marker gene and a transcription termination sequence (stop cassette) flanked by *FRT*s in a head-to-tail arrangement. Expression of *flippase* by heat-shock induction or under control of a tissue-specific promoter can then induce recombination between the two *FRT*s, which leads to excision of the cassette, and in consequence the promoter can directly drive the expression of the gene of interest (Fig. 5.3). The flip-out strategy was further used by two independent research groups to develop an elegant system for domain-specific ectopic gene expression during early stages of *Drosophila* embryogenesis when binary expression systems cannot be used (Kosman and Small, 1997; Wimmer *et al.*, 1997). This system is superior to the original *flip-out* system in the use of region-specific enhancer/promoters rather than a constitutive promoter for a more defined expression of the gene, and the inclusion of a marker gene in the *flip-out* cassette that enables early selection for lines that would later express the gene of interest
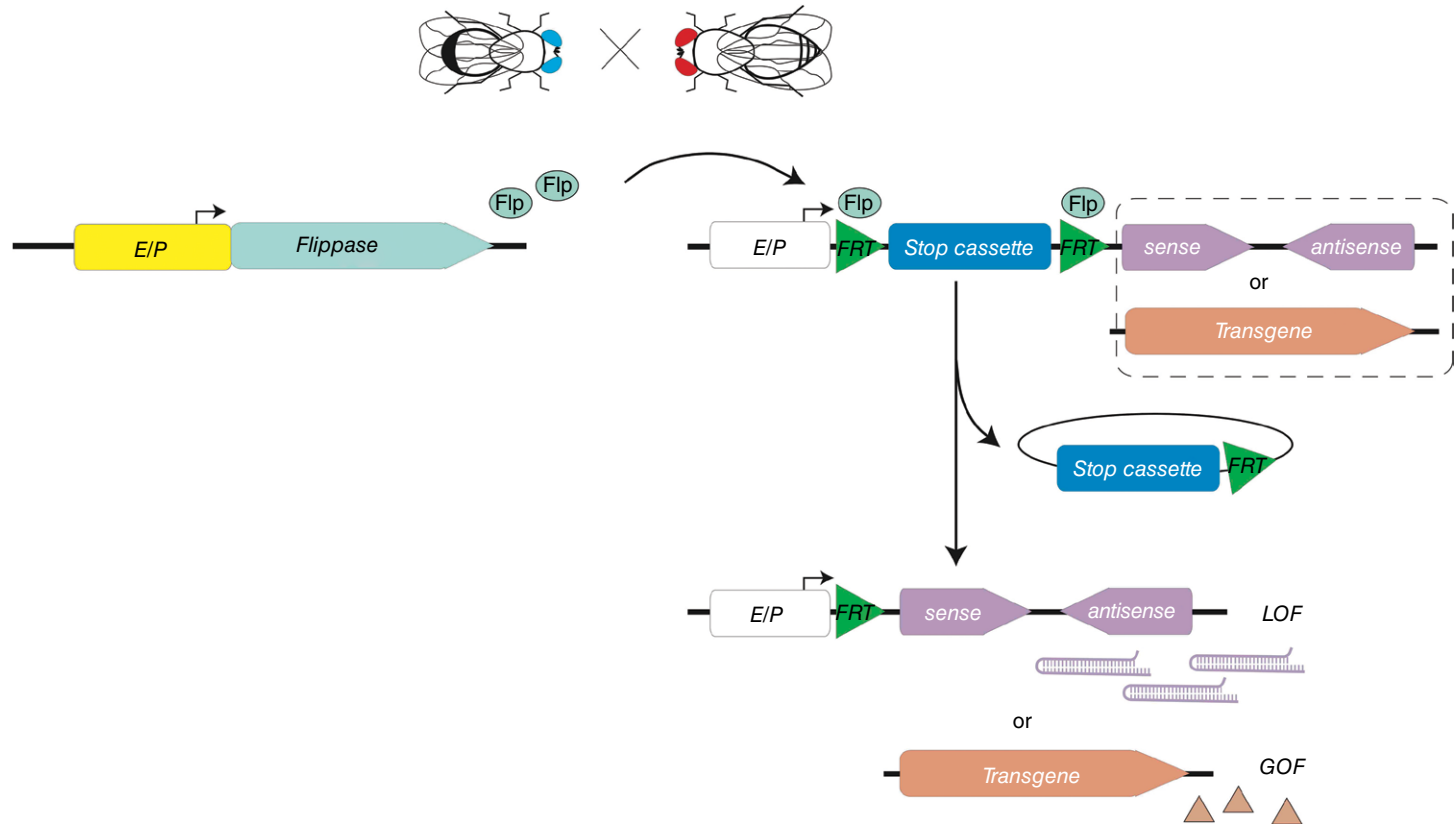
Fig. 5.3. Mosaic activation of transgenes (Struhl and Basler, 1993; Wimmer *et al.*, 1997). To spatially and temporally confine expression of a transgene, a flip-out cassette containing an *RNA PolII* terminator (Stop cassette) flanked by two identical *FRT* sites is placed between the enhancer/promoter (E/P) and the gene to be expressed. In this case the gene is not active, since the transcription will be terminated by the cassette. The transgene is activated by a cross between flies harbouring the gene containing the flip-out cassette and flies carrying a *flippase* driver construct that leads to the expression of *flippase* in a particular tissue or a stage using an independent tissue-specific E/P or heat-shock inducible promoter. With this system, both loss-of-function (LOF) and gain-of-function (GOF) situations can be generated, by either mis- or overexpression of a transgene or the expression of a hairpin construct producing dsRNA causing RNA interference, respectively.

in the expected way. Moreover, the use of another promoter driving *flippase* expression could further refine the rearrangement of the transgene in a configuration ready for expression in the right tissue and at the right time or stage.

In insect biotechnology, toxic effector molecules often need to be expressed conditionally as well as tissue- and stage-specifically to develop transgenic pest control strategies suitable for SIT. Binary systems such as *tet-off* have been intensively used to build different transgenic systems to drive the expression of effector molecules indirectly and in a controlled fashion (Heinrich and Scott, 2000; Horn and Wimmer, 2003) (see Scott *et al.*, Chapter 17; Handler and Schetelig, Chapter 21; Morrison, Chapter 23, this volume). In some cases, due to leakiness of basal promoters used in the effector construct downstream to the upstream activation sequence of the respective system, no functional transgenic lines can be obtained. Here the *flip-out* system can offer a great solution to generate respective lines (Eckermann *et al.*, 2014). SSR-mediated small excision or inversion strategies have also been successful using the Cre-*Lox* system in *Ae. aegypti* (Jasinskiene, 2003) or the *attP/attB* φC31 system in *B. mori* (Wang *et al.*, 2021).

Modification and stabilization of transgenes

Several class II DNA transposable elements – such as *P* element*, Minos, mariner, Hermes*, or *piggyBac* – have been identified and adopted as gene vectors. Intact functional transposable elements consist of a gene encoding the transposase necessary for its movement flanked by inverted terminal repeat (*ITR*) sequences. For the element to move, the transposase recognizes and binds the *ITR* and catalyses a cut-and-paste reaction of the element into a new genomic target (Rubin and Spradling, 1982). This information was exploited to build non-autonomous transposon-based gene vectors (TBVs) that lack the transposase and therefore cannot move by themselves, and the respective transposase is provided in *trans* from a helper plasmid under constitutive or inducible promoters. Genetic engineering of model and non-model

insects is routinely done by such non-autonomous TBVs (see O'Brochta, Chapter 1, this volume).

After integration into the genome, TBVs can be remobilized, if exposed to their own or closely related transposases, which might lead to loss of the transgene or to reintegration at another genomic position affected by different position effects. This instability issue raised concerns for biotechnological pest control strategies, for which millions of transgenic insects need to be produced in large facilities for field release. Loss or mobilization of the transgene to new genomic loci compromises the efficiency of the strain for the intended purpose. To address this issue, several transgene stabilization strategies were developed based on the fact that both the 5′ and 3′ *ITRs* are necessary for transposition (Handler *et al.*, 2004; Horn and Handler, 2005; Dafa'alla *et al.*, 2006). The best transgene immobilization scenario would be to delete both the 5′ and 3′ *ITRs* (Fig. 5.4A). This has been achieved in *C. capitata* by engineering a complex *piggyBac* vector. Basically, the transgene of interest along with a transformation marker M2 was flanked by two intact *piggyBac* vectors harbouring two additional different transformation markers M1 and M3. When this vector was microinjected along with the transposase helper, only F1 individuals that had the three markers were chosen, exposed again to transposase activity, and screened for progeny that displayed only the marker M2 associated with the transgene of interest (Fig. 5.4A) (Dafa'alla *et al.*, 2006). The major limitations of the system are the size of the construct and the different possible outcomes, which both contribute to a very low efficiency of germline transformation.

A similar approach has been developed in *D. melanogaster*, but it deletes only one *ITR* and thus renders the vector unable to move (Fig. 5.4B). The TBV was arranged in such a way that it contained two different transformation markers and an alternative internal 5′ *ITR*. Initially the construct was integrated and flies that display both markers were selected. Those flies were then exposed to transposase activity and only flies that expressed M1 were chosen. Molecular
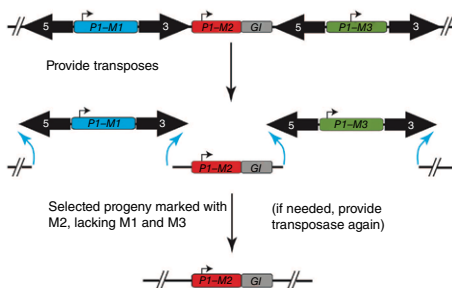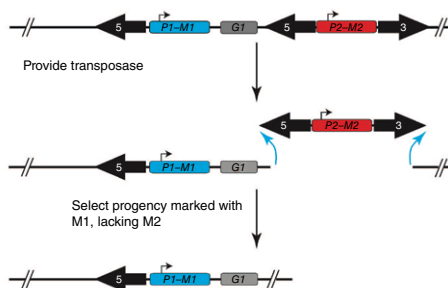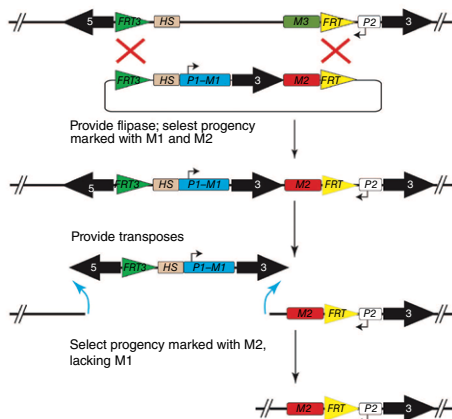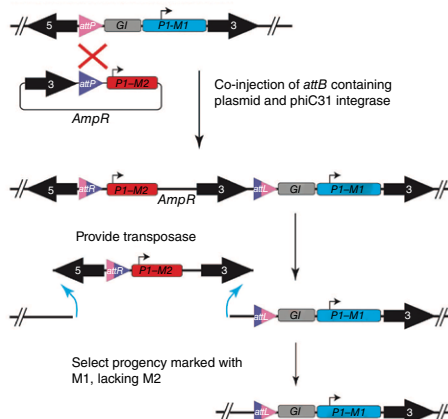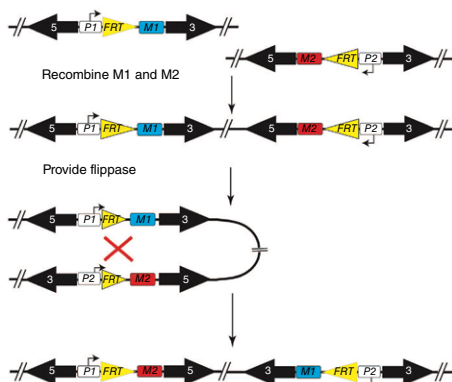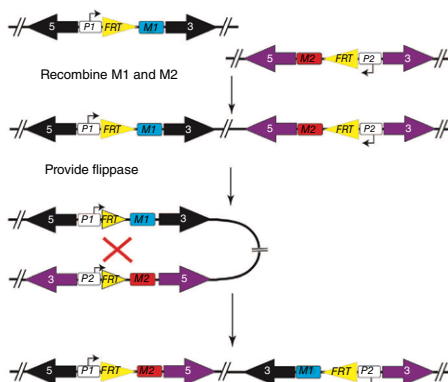
Fig. 5.4. Strategies for transgene stabilization. Blue arrow indicates transposase-induced remobilization and loss of transgene parts. The red cross stands for recombination. **(A–D)** Strategies that rely on removal of one or both transposon inverted terminal repeats (*ITR*s) rendering the transgene stable.

characterization confirmed the deletion of the internal 5′ *ITR*, marker M2 and the 3′ *ITR*, leaving behind only the outside 5′ *ITR*, marker M1 and the transgene. The stability of the transgene was verified by exposing the flies again to transposase activity (Handler *et al*., 2004). However, this approach suffers from the same limitations described above in a decrease in the efficiency of germline transformation.

To overcome this negative effect on germline transformation efficiency as well as the size limitation, a new strategy based on the use of Flp-*FRT* SSR system was developed in *D. melanogaster* (Fig. 5.4C). The original TBV contained a transformation marker M3 flanked by two diverse *flippase* recognition target sites (*FRT* and *FRT3*). By RMCE, an additional 3′ *ITR* flanked by two independent markers M1 and M2 was integrated, thereby replacing the original marker M3. In a next step the flies were again exposed to transposase activity and only flies that displayed marker M2 were selected. Molecular characterization revealed that the 5′ *ITR* was removed along with marker M1 and the 3′ *ITR*, rendering the remaining transgene stable (Horn and Handler, 2005). A related strategy was also developed for the medfly *C. capitata* for modification and stabilization of transgenes (Fig. 5.4D) using a single φC31 *attP* site, which was included in the TBV. Transgenic flies were then subjected to integration of a donor plasmid containing an *attB* site, a second transformation marker M2 and 3′ *ITR*. Flies showing the successful integration of the donor plasmid indicated by the presence of both transformation markers were then exposed to transposase activity. In the next generation, flies that

only expressed the initial marker M1 were shown to have the 5′ *ITR* removed along with marker M2, the introduced 3′ *ITR* and the integrated plasmid backbone. The left-behind marker M1 and the transgene were then proven to be stabilized (Schetelig *et al*., 2009). All of the above strategies for transgene stabilization were based on deletion of one or both of the vector *ITR*s by inclusion of extra *ITR*s in the original TBV or by a second step using SSR technologies. This can also be achieved using CRISPR/Cas9 to induce DSBs within the integrated TBV and the use or HDR or NHEJ pathways to introduce the additional *ITR* along with a marker to enable screening. The *ITR*s of a TBV can also be removed by introducing, in a second step (mediated, for example, by *attP/attB* φC31 integration), endonuclease recognition sites to generate DSBs. Along with these sites short copies of the TBV-flanking genomic regions are also inserted. The induction of DSBs then causes HDR between the homologous sequences, thus removing the TBV ends (Tkachuk *et al*., 2011).

A different approach for transgene stabilization was developed in *D. melanogaster*, which is based on recombination of two linked transgenes each harbouring a single *FRT* site in a head-to-head arrangement in respect to each other (Figs 5.4E and 5.4F) (Schetelig *et al*., 2011). The transgenic lines were independently generated using either *piggyBac-* or *Hermes*-based vectors and were chosen to be on the same chromosome. Meiotic recombination was exploited to bring the two transgenes together as a linked group on one chromosome. The vectors are built in such a way that the *FRT* is inserted between the promoter and the coding

Fig. 5.4. Continued.

(A) A *piggyBac* vector contains additional internal 5′ and 3′ *ITR*s in an arrangement that facilitates subsequent removal of all *piggyBac ITR*s (Dafa'alla *et al*., 2006). **(B)** Only an additional internal 5′ *piggyBac ITR* is included, which enables removal of the 3′ *piggyBac ITR* (Handler *et al*., 2004). **(C)** An additional internal *piggyBac ITR* is introduced by RMCE (Horn and Handler, 2005) or **(D)** integration in an *attP* site (Schetelig *et al*., 2009), which enable subsequent removal of the original 5′ *piggyBac ITR* rendering the transgene stable. **(E, F)** Transgenes stability is achieved by intrachromosomal recombination between two *FRT* sites in two different transgene integrations located on the same chromosome in a head-to-head arrangement. The two transgenes are either of the same **(E)** or different **(F)** transposon origin. Rearrangement leads to two stable transgenes, each bordered by either two 3′ *ITR*s or two 5′ *ITR*s (reviewed in Schetelig *et al*., 2011).

sequence of the transformation marker. The promoters and the transformation markers are different for each of the two linked insertions. Exposure of such lines to flippase activity leads to an inversion of the DNA between the two identical *FRTs* that are oriented in a head-to-head fashion (Fig. 5.5A) (see also 'Chromosome engineering by site-specific recombination' in section 5.3.4, below). This chromosomal rearrangement is identified by the exchange of the pattern of the two transformation markers. As a result, the recombined integrations then carry either two 3′ *ITRs* or two 5′ *ITRs* of one transposon (e.g. *piggyBac*)
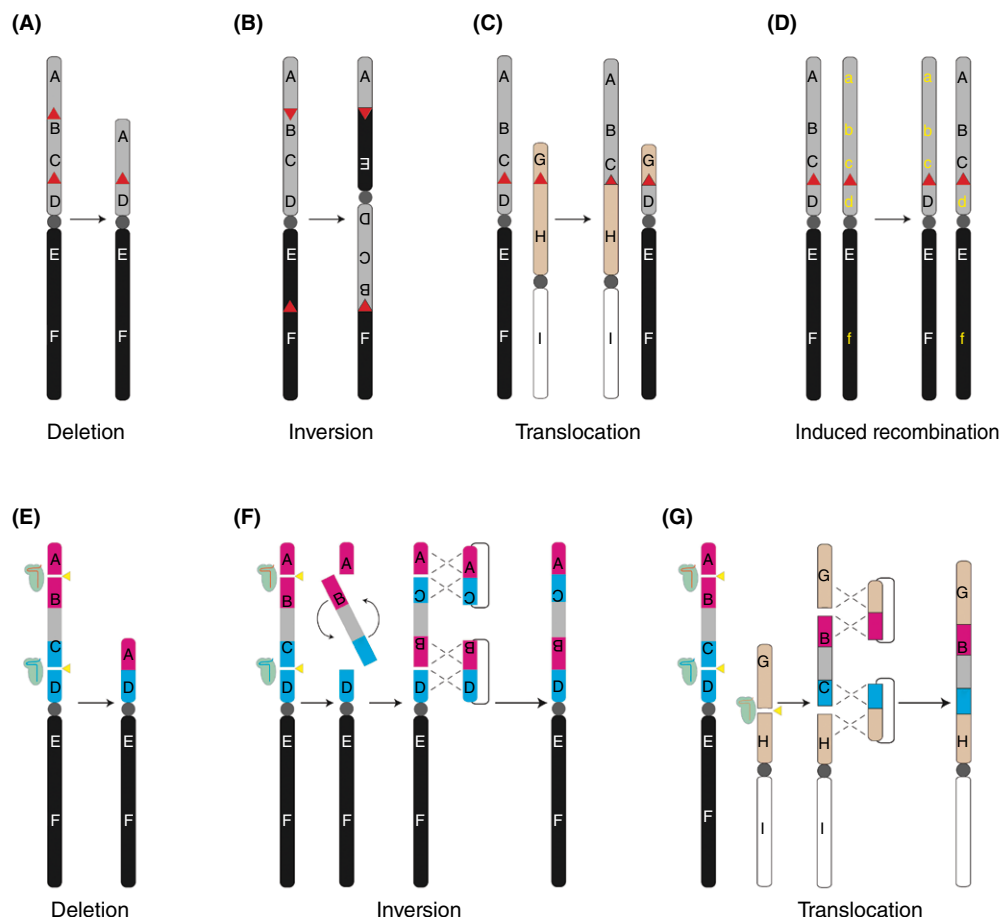
Fig. 5.5. Chromosomal engineering. Red triangles stand for *FRT* sites, yellow triangles indicate CRISPR/Cas9 target sites. (A) Flippase-mediated recombination results in deletion of a chromosomal part flanked by two *FRT* sites in a head-to-tail arrangement (Golic and Lindquist, 1989; Golic, 1991). **(B)** Recombination between *FRT* sites in head-to-head orientation leads to a chromosomal inversion (Golic and Lindquist, 1989; Golic, 1991). **(C)** Translocation of chromosomal material between non-homologous chromosomes harbouring *FRT* sites (Beumer *et al*., 1998). **(D)** Induced mitotic recombination between homologous chromosomes (Golic, 1991). **(E)** CRISPR/Cas9-induced chromosomal deletion achieved by induction of two DSBs flanking the region to be deleted (He *et al*., 2015; Cullot *et al*., 2019). **(F)** Chromosomal inversion following CRISPR/Cas9-induced DSBs and guided by provision of homology repair templates (Iwata *et al*., 2016). **(G)** Similar to guided chromosomal inversions, chromosomal translocations can be achieved by induction of two DSBs flanking the DNA stretch to be translocated, a single DSB where the fragment should be translocated to and two homology repair templates to guide the precise translocation (Jiang *et al*., 2016).

(Fig. 5.4E) or different transposons (e.g. *piggyBac* and *Hermes*) (Fig. 5.4F), which are no longer able to move when exposed to the respective transposase enzymes.

The combination of the two Cre-*Lox* and Flp-*FRT* SSR systems also enables the co-placement of two transgenic alleles by transposon-based integration and successive independent removal of either single allele for comparison of the two different alleles at the same genomic position (Siegal and Hartl, 1996). Successive rounds of SSR with different systems can change again and again the transgenic locus for additional modifications (Huang *et al*., 2009, 2011). The CRISPR/Cas9-based HDR strategy has also been used to modify previously integrated transgenes. In the flour beetle *T. castaneum*, an *EGFP* marker was exchanged with a *DsRed* marker (Gilles *et al*., 2015); in *C. capitata*, an *EGFP* marker was converted into a blue fluorescent marker (Aumann *et al*., 2018); and in the invasive fruit pest *D. suzukii*, a *piggyBac* insertion was precisely modified by knock-in of an *EGFP*-based body marker and the promoter of the spermatogenesis specific gene *beta-2-tubulin* to drive the expression of the pre-inserted *DsRed* specifically in the testes (Ahmed *et al*., 2019).

5.3.4 Gene locus-directed chromosome modification: deletions, inversions and translocations

Custom or controlled chromosomal modifications such as deletions, inversions and translocations are collectively called chromosome engineering (Carlson and Largaespada, 2005). All can happen spontaneously due to errors during cell division or exposure to radiation and in these cases are referred to as chromosomal aberrations or anomalies, since they are the base of many genetic diseases, including cancer (Preston, 2014). Induction of inversions and translocation by radiation was an important tool in early genetic studies (Kaufmann, 1939; Abrahamson, 1961). Molecular biology tools such as SSR (Golic and Lindquist, 1989) or induced DSBs can also be used to generate chromosomal rearrangements.

Chromosome engineering by site-specific recombination

SSR using the Cre-*Lox* or Flp-*FRT* systems requires the presence of two identical copies of the respective system in the genome of the target insect. Exposure to the recombinase causes recombination between the two target sites and the outcome is dependent on the exact position (on the same or different chromosomes) and arrangement (head-to-head, head-to-tail) of the RRSs (Fig. 5.5A–D) (Golic and Lindquist, 1989; Golic, 1991).

Chromosomal deletions are an important genetic tool to map mutations to defined regions of the chromosome (Golic and Lindquist, 1989; Golic, 1991; Cook *et al*., 2012). In addition, deletions can generate hemizygous situations leading to enhanced or reduced phenotypes arising from mutant alleles. Traditionally, deletions were achieved by chemical mutagens and irradiation or by imprecise excision of *P* elements and other transposon-based vectors (Preston *et al*., 1996; Huet *et al*., 2002). Also, the Flp-*FRT* SSR system has offered an attractive alternative to produce regional chromosomal deficiencies. Meiotic recombination can combine two *FRT* sites on the same chromosome. In case they are in a head-to-tail arrangement, an induced SSR can lead to the deletion of the intervening region (Fig. 5.5A). This can be highly efficient (up to 100%) when the two *FRT* sites are only a few kilobases apart. However, the efficiency drops to a few per cent with the increase of the size to the range of megabases. This system was used in large-scale chromosomal deletion projects in *D. melanogaster*, generating sets of deletion stocks covering almost all of the genome (Ryder *et al*., 2004, 2007).

To engineer chromosomal inversions based on site-specific recombination, two recombinase recognition target sites need to be combined on the same chromosome in a head-to-head orientation (Fig. 5.5B). Such inversions have been generated in *D. melanogaster* using the Flp-*FRT* SSR system as a strategy to stabilize two transgenes that harbour *FRT*s (Figs. 5.4E and 5.4F) (Schetelig *et al*., 2011). These inversions also function as meiotic recombination suppressors and

can be used as partial balancer chromosomes (Dobzhansky and Epling, 1948).

Exchange of chromosomal segments between non-homologous chromosomes, known as translocation, has been used as a tool to study the mechanisms of segregation of meiotic chromosomes and analysis of aneuploidy in *Drosophila* (Beumer *et al.*, 1998). Moreover, translocations were proposed as vector control strategy to drive specific traits in wild populations such as refractoriness of *Anopheles* to malaria parasites and thus breaking the transmission cycle (Gayathri Devi and Shetty, 1992; Davis *et al.*, 2001). Autosome to sex chromosome translocation is the base of the most elegant and most widely used genetic sexing strain for SIT of the medfly *C. capitata* (Robinson and Van Heemert, 1982; Franz *et al.*, 1994). Traditionally such translocations were induced by radiation (Kaufmann, 1939), which complicates the analysis of the outcome of the translocation due to radiation-induced damage. The Flp-*FRT* SSR system has been adopted to introduce defined chromosomal translocations between heterologous chromosomes in *D. melanogaster* (Beumer *et al.*, 1998) by recombining chromosomes that each contain *FRT* sites (Fig. 5.5C).

Among the most powerful tools for *Drosophila* developmental genetics is mosaic analysis to study cell autonomy, lineage tracing and mechanisms of embryonic and adult patterning. Genetic mosaics have been generated using different strategies, including cell or nuclear transplantation, the ring-X chromosome technique, or induction of mitotic recombination by ionizing radiation. The use of the yeast Flp-*FRT* SSR system to induce mitotic recombination between homologous chromosomes offered an ingenious alternative to previous technologies and overcame the shortcomings of ionizing irradiation, such as unintended developmental defects and low efficiency (Fig. 5.5D). Linked to different cell autonomous markers, the Flp-*FRT* SSR system has been well established in *Drosophila* for analysing embryonic, adult and germline clones (Dang and Perrimon, 1992; Xu and Rubin, 1993). The use of a germline-specific dominant female sterile (*DFS*) mutation (Wieschaus *et al.*,

1981; Perrimon and Gans, 1983) has enabled, in addition to the analysis of recessive female sterile mutations, the detection of the maternal effect of recessive zygotic lethal mutations. The *DFS* system initially relied on radiation-induced mitotic recombination until it was demonstrated that induced mitotic recombination can be achieved in both the soma and the germ cells of *Drosophila* by the yeast Flp-*FRT* SSR system (Golic, 1991). Later, an autosomal *FLP-DFS* technique was developed by linking the *DFS* mutation to *FRT*s on the second and third chromosomes (Chou and Perrimon, 1996).

CRISPR/Cas9-mediated chromosome engineering

High-resolution chromosomal rearrangements can also be engineered using CRISPR/Cas9 genome editing tools (Fig. 5.5E–G). Rearrangements can be achieved by targeting repetitive elements in the genome and rely on NHEJ or HDR using *cis* or *trans* uncut elements as repair templates to put different fragments together, in the hope of obtaining balanced and heritable reshaped genomes (Fleiss *et al.*, 2019). However, to generate site-specific chromosomal arrangements, Cas9 offers the ultimate tool to induce defined DNA DSBs and guide the arrangement using repair templates that dictate the outcome. The system was used to induce precise chromosome deletions by using two guides demarcating the targeted region (Fig. 5.5E) (He *et al.*, 2015; Cullot *et al.*, 2019). It has also been successfully used in the nematode worm *Caenorhabditis elegans* to engineer balancer chromosomes by inversions (Fig. 5.5F) (Iwata *et al.*, 2016) and in embryonic stem cells to induce site-specific translocations (Fig. 5.5G) (Jiang *et al.*, 2016). For biotechnologically improved pest control, scientists are using CRISPR/Cas9-mediated mutagenesis to copy selectable marker mutations known from sexing strains of the medfly *C. capitata* and transfer these to other pest species by gene editing (Ward *et al.*, 2021). In a first step, CRISPR/Cas9 has been used to induce LOF mutations in the *white pupae* gene, which is essential for the normal brown pupal colour. A wild-type rescue version of

the recessive marker mutation could then be translocated to the male sex chromosome to rescue the phenotype in males only. This should be achievable using a CRISPR/Cas9-induced chromosomal translocation by introduction of two DSBs encompassing the wild-type allele and a single DSB on the Y chromosome. The use of a repair template should then guide the translocation to the Y chromosome (Fig. 5.5G). In mass production, brown male pupae could be readily separated from white female pupae and then sterilized by radiation before release.

5.4   Conclusions

SSR based on site-specific recombinases, which bind to short recognition sequences, as well as the site-specific introduction of DSBs can be used to cause recombination-mediated genome modifications leading to gene locus-directed transgene integration and modification. The induction of CRISPR/Cas9-mediated DSBs to cause integration is a very useful tool when suitable genomic loci are identified that support transgene expression or when specific genes are targeted. However, for many insects, the information on suitable genomic target sites for successful expression of transgenic constructs is very limited. Therefore, transposon-mediated random genome insertion can first be used to introduce SSR target sites for integration, RMCE, or chromosome engineering. The most usable sites can then be identified by characterization and evaluation for suitable and stable transgene expression at sufficient levels and with minimal position effects. Once such chromosomal positions have been identified, all the described recombination technologies can then be used to further modify the transgenes in whatever intended way.

References

Abrahamson, S. (1961) Chromosome rearrangements induced by X-rays in immature germ cells of *Drosophila*. *Nature* 191, 523–524. doi: 10.1038/191523b0

Ahmed, H.M.M., Hildebrand, L. and Wimmer, E.A. (2019) Improvement and use of CRISPR/Cas9 to engineer a sperm-marking strain for the invasive fruit pest *Drosophila suzukii*. *BMC Biotechnology* 19, 85. doi: 10.1186/s12896-019-0588-5

Ahmed, H.M.M., Heese, F. and Wimmer, E.A. (2020) Improvement on the genetic engineering of an invasive agricultural pest insect, the cherry vinegar fly, *Drosophila suzukii*. *BMC Genetics* 21, 139. doi: 10.1186/s12863-020-00940-5

Aizawa, S. (1995) [Homologous recombination for gene targeting]. *Tanpakushitsu Kakusan Koso* 40, 2013–2016.

Amenya, D.A., Bonizzoni, M., Isaacs, A.T., Jasinskiene, N., Chen, H. *et al.* (2010) Comparative fitness assessment of *Anopheles stephensi* transgenic lines receptive to site-specific integration. *Insect Molecular Biology* 19, 263–269. doi: 10.1111/j.1365-2583.2009.00986.x

Andreas, S., Schwenk, F., Küter-Luks, B., Faust, N. and Kühn, R. (2002) Enhanced efficiency through nuclear localization signal fusion on phage PhiC31-integrase: activity comparison with Cre and FLPe recombinase in mammalian cells. *Nucleic Acids Research* 30, 2299–2306. doi: 10.1093/nar/30.11.2299

Auer, T.O., Duroure, K., De Cian, A., Concordet, J.-P. and Del Bene, F. (2014) Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. *Genome Research* 24, 142–153. doi: 10.1101/gr.161638.113

Aumann, R.A., Schetelig, M.F. and Häcker, I. (2018) Highly efficient genome editing by homology-directed repair using Cas9 protein in *Ceratitis capitata*. *Insect Biochemistry Molecular Biology* 101, 85–93. doi: 10.1016/j.ibmb.2018.08.004

Aumann, R.A., Häcker, I. and Schetelig, M.F. (2020) Female-to-male sex conversion in *Ceratitis capitata* by CRISPR/Cas9 HDR-induced point mutations in the sex determination gene *transformer-2*. *Scientific Reports* 10, 18611. doi: 10.1038/s41598-020-75572-x

Baer, A. and Bode, J. (2001) Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes. *Current Opinion in Biotechnology* 12, 473–480. doi: 10.1016/S0958-1669(00)00248-2

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P. *et al.* (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315, 1709–1712. doi: 10.1126/science.1138140

Bassett, A.R., Tibbit, C., Ponting, C.P. and Liu, J.-L. (2013) Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Reports* 4, 220–228. doi: 10.1016/j.celrep.2013.06.020

Basu, S., Aryan, A., Overcash, J.M., Samuel, G.H., Anderson, M.A.E. *et al.* (2015) Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in *Aedes aegypti*. *Proceedings of the National Academy of Sciences USA* 112, 4038–4043. doi: 10.1073/pnas.1502370112

Bateman, J.R., Lee, A.M. and Wu, C.T. (2006) Site-specific transformation of *Drosophila* via φC31 integrase-mediated cassette exchange. *Genetics* 173, 769–777. doi: 10.1534/genetics.106.056945

Belteki, G., Gertsenstein, M., Ow, D.W. and Nagy, A. (2003) Site-specific cassette exchange and germline transmission with mouse ES cells expressing *phiC31* integrase. *Nature Biotechnology* 21, 321–324. doi: 10.1038/nbt787

Beumer, K.J., Pimpinelli, S. and Golic, K.G. (1998) Induced chromosomal exchange directs the segregation of recombinant chromatids in mitosis of *Drosophila*. *Genetics* 150, 173–188.

Beumer, K.J., Trautman, J.K., Bozas, A., Liu, J.-L., Rutter, J. *et al.* (2008) Efficient gene targeting in *Drosophila* by direct embryo injection with zinc-finger nucleases. *Proceedings of the National Academy of Sciences USA* 105, 19821–19826. doi: 10.1073/pnas.0810475105

Bibikova, M., Golic, M., Golic, K.G. and Carroll, D. (2002) Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. *Genetics* 161, 1169–1175. doi: 10.1093/genetics/161.3.1169

Bischof, J., Maeda, R.K., Hediger, M., Karch, F. and Basler, K. (2007) An optimized transgenesis system for *Drosophila* using germ-line-specific φC31 integrases. *Proceedings of the National Academy of Sciences USA* 104, 3312–3317. doi: 10.1073/pnas.0611511104

Bode, J., Schlake, T., Iber, M., Schübeler, D., Seibler, J. *et al.* (2005) The transgeneticists toolbox: novel methods for the targeted modification of eukaryotic genomes. *Biological Chemistry* 381, 801–813. doi: 10.1515/BC.2000.103

Bouhassira, E.E., Westerman, K. and Leboulch, P. (1997) Transcriptional behavior of LCR enhancer elements integrated at the same chromosomal locus by recombinase-mediated cassette exchange. *Blood* 90, 3332–3344. doi: 10.1182/blood.V90.9.3332

Brand, A.H., Manoukian, A.S. and Perrimon, N. (1994) Ectopic expression in *Drosophila*. In: Goldstein, L.S.B. and Fyrberg, E.A. (eds) *Methods in Cell Biology*, Vol. 44. Academic Press, Cambridge, Massachusetts, pp. 635–654. doi: 10.1016/S0091-679X(08)60936-X

Broach, J.R., Guarascio, V.R. and Jayaram, M. (1982) Recombination within the yeast plasmid 2mu circle is site-specific. *Cell* 29, 227–234. doi: 10.1016/0092-8674(82)90107-6

Brouns, S.J.J., Jore, M.M., Lundgren, M., Westra, E.R., Slijkhuis, R.J.H. *et al.* (2008) Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* 321, 960–964. doi: 10.1126/science.1159689

Campbell, A.M. (1963) Episomes. In: Caspari, E.W. and Thoday, J.M. (eds) *Advances in Genetics*. Academic Press, Cambridge, Massachusetts, pp. 101–145. doi: 10.1016/S0065-2660(08)60286-2

Carlson, C.M. and Largaespada, D.A. (2005) Insertional mutagenesis in mice: new perspectives and tools. *Nature Reviews Genetics* 6, 568–580. doi: 10.1038/nrg1638

Carroll, D. (2013) Genetic recombination. In: Maloy, S. and Hughes, K. (eds) *Brenner's Encyclopedia of Genetics* (2nd edn). Academic Press, San Diego, California, pp. 277–280. doi: 10.1016/B978-0-12-374984-0.00627-6

Champer, J., Liu, J., Oh, S.Y., Reeves, R., Luthra, A. *et al.* (2018) Reducing resistance allele formation in CRISPR gene drive. *Proceedings of the National Academy of Sciences USA* 115, 5522–5527.

Chou, T.B. and Perrimon, N. (1996) The autosomal FLP-DFS technique for generating germline mosaics in *Drosophila melanogaster*. *Genetics* 144, 1673–1679. doi: 10.1093/genetics/144.4.1673

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R. *et al.* (2013) Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* 339, 819–823. doi: 10.1126/science.1231143

Cook, R.K., Christensen, S.J., Deal, J.A., Coburn, R.A., Deal, M.E. *et al.* (2012) The generation of chromosomal deletions to provide extensive coverage and subdivision of the *Drosophila melanogaster* genome. *Genome Biology* 13, R21. doi: 10.1186/gb-2012-13-3-r21

Craig, N.L. (1988) The mechanism of conservative site-specific recombination. *Annual Review of Genetics* 22, 77–105. doi: 10.1146/annurev.ge.22.120188.000453

Cristea, S., Freyvert, Y., Santiago, Y., Holmes, M.C., Urnov, F.D. *et al.* (2013) In vivo cleavage of transgene donors promotes nuclease-mediated targeted integration. *Biotechnology and Bioengineering* 110, 871–880. doi: 10.1002/bit.24733

Cullot, G., Boutin, J., Toutain, J., Prat, F., Pennamen, P. *et al.* (2019) CRISPR-Cas9 genome editing induces megabase-scale chromosomal truncations. *Nature Communications* 10, 1136. doi: 10.1038/s41467-019-09006-2

Dafa'alla, T.H., Condon, G.C., Condon, K.C., Phillips, C.E., Morrison, N.I. *et al.* (2006) Transposon-free insertions for insect genetic engineering. *Nature Biotechnology* 24, 820–821. doi: 10.1038/nbt1221

Dang, D.T. and Perrimon, N. (1992) Use of a yeast site-specific recombinase to generate embryonic mosaics in *Drosophila*. *Developmental Genetics* 13, 367–375.

Davis, S., Bax, N. and Grewe, P. (2001) Engineered underdominance allows efficient and economical introgression of traits into pest populations. *Journal of Theoretical Biology* 212, 83–98. doi: 10.1006/jtbi.2001.2357

Deltcheva, E., Chylinski, K., Sharma, C.M., Gonzales, K., Chao, Y. *et al.* (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 471, 602–607. doi: 10.1038/nature09886

Ding, S., Wu, X., Li, G., Han, M., Zhuang, Y. and Xu, T. (2005) Efficient transposition of the *piggyBac* (PB) transposon in mammalian cells and mice. *Cell* 122, 473–483. doi: 10.1016/j.cell.2005.07.013

Dobzhansky, T. and Epling, C. (1948) The suppression of crossing over in inversion heterozygotes of *Drosophila pseudoobscura*. *Proceedings of the National Academy of Sciences USA* 34, 137–141. doi: 10.1073/pnas.34.4.137

Eckermann, K.N., Dippel, S., KaramiNejadRanjbar, M., Ahmed, H.M., Curril, I.M. and Wimmer, E.A. (2014) Perspective on the combined use of an independent transgenic sexing and a multifactorial reproductive sterility system to avoid resistance development against transgenic Sterile Insect Technique approaches. *BMC Genetics* 15, S17.

Eckermann, K.N., Ahmed, H.M.M., KaramiNejadRanjbar, M., Dippel, S., Ogaugwu, C.E. *et al.* (2018) Hyperactive *piggyBac* transposase improves transformation efficiency in diverse insect species. *Insect Biochemistry Molecular Biology* 98, 16–24.

Farnworth, M.S., Eckermann, K.N., Ahmed, H.M.M., Mühlen, D.S., He, B. and Bucher, G. (2020a) The red flour beetle as model for comparative neural development: genome editing to mark neural cells in *Tribolium* brain development. In: Sprecher, S.G. (ed.) *Brain Development: Methods and Protocols, Methods in Molecular Biology*. Springer, New York, pp. 191–217. doi: 10.1007/978-1-4939-9732-9_11

Farnworth, M.S., Eckermann, K.N. and Bucher, G. (2020b) Sequence heterochrony led to a gain of functionality in an immature stage of the central complex: a fly–beetle insight. *PLoS Biology* 18, e3000881. doi: 10.1371/journal.pbio.3000881

Fernandes, F., Vidigal, J., Dias, M.M., Prather, K.L.J., Coroadinha, A.S. *et al.* (2012) Flipase-mediated cassette exchange in Sf9 insect cells for stable gene expression. *Biotechnology and Bioengineering* 109, 2836–2844. doi: 10.1002/bit.24542

Fleiss, A., O'Donnell, S., Fournier, T., Lu, W., Agier, N. *et al.* (2019) Reshuffling yeast chromosomes with CRISPR/Cas9. *PLoS Genetics* 15, e1008332. doi: 10.1371/journal.pgen.1008332

Fogg, P.C.M., Younger, E., Fernando, B.D., Khaleel, T., Stark, W.M. and Smith, M.C.M. (2018) Recombination directionality factor gp3 binds ϕC31 integrase via the zinc domain, potentially affecting the trajectory of the coiled-coil motif. *Nucleic Acids Research* 46, 1308–1320. doi: 10.1093/nar/gkx1233

Franz, A.W., Jasinskiene, N., Sanchez-Vargas, I., Isaacs, A.T., Smith, M.R. *et al.* (2011) Comparison of transgene expression in *Aedes aegypti* generated by mariner *Mos1* transposition and *ΦC31* site-directed recombination. *Insect Molecular Biology* 20, 587–598. doi: 10.1111/j.1365-2583.2011.01089.x.

Franz, G., Gencheva, E. and Kerremans, P. (1994) Improved stability of genetic sex-separation strains for the Mediterranean fruit fly, *Ceratitis capitata*. *Genome* 37, 72–82. doi: 10.1139/g94-009

Friedman, D.I. (1992) Interaction between bacteriophage *lambda* and its *Escherichia coli* host. *Current Opinion in Genetics & Development* 2, 727–738. doi: 10.1016/s0959-437x(05)80133-9

Gasiunas, G., Barrangou, R., Horvath, P. and Siksnys, V. (2012) Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences USA* 109, E2579–E2586. doi: 10.1073/pnas.1208507109

Gayathri Devi, K. and Shetty, N.J. (1992) Chromosomal translocations and inherited semisterility in the malaria vector *Anopheles stephensi* Liston. *Journal of Communicative Diseases* 24, 70–74.

Gilles, A.F., Schinko, J.B. and Averof, M. (2015) Efficient CRISPR-mediated gene targeting and transgene replacement in the beetle *Tribolium castaneum*. *Development* 142, 2832–2839. doi: 10.1242/dev.125054

Golic, K.G. (1991) Site-specific recombination between homologous chromosomes in *Drosophila*. *Science* 252, 958–961. doi: 10.1126/science.2035025

Golic, K.G. and Lindquist, S. (1989) The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. *Cell* 59, 499–509. doi: 10.1016/0092-8674(89)90033-0

Gong, W.J. and Golic, K.G. (2003) Ends-out, or replacement, gene targeting in *Drosophila*. *Proceedings of the National Academy of Sciences USA* 100, 2556–2561. doi: 10.1073/pnas.0535280100

Gratz, S.J., Cummings, A.M., Nguyen, J.N., Hamm, D.C., Donohue, L.K. *et al.* (2013) Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. *Genetics* 194, 1029–1035. doi: 10.1534/genetics.113.152710

Grindley, N.D.F., Whiteson, K.L. and Rice, P.A. (2006) Mechanisms of site-specific recombination. *Annual Review of Biochemistry* 75, 567–605. doi: 10.1146/annurev.biochem.73.011303.073908

Groth, A.C., Fish, M., Nusse, R. and Calos, M.P. (2004) Construction of transgenic *Drosophila* by using the site-specific integrase from phage *phiC31*. *Genetics* 166, 1775–1782. doi: 10.1534/genetics.166.4.1775

Häcker, I., Harrell II, R.A., Eichner, G., Pilitt, K.L., O'Brochta, D.A. *et al.* (2017) Cre/lox-recombinase-mediated cassette exchange for reversible site-specific genomic targeting of the disease vector, *Aedes aegypti*. *Scientific Reports* 7, 43883. doi: 10.1038/srep43883

Haghighat-Khah, R.E., Scaife, S., Martins, S., John, O.S., Matzen, K.J. *et al.* (2015) Site-specific cassette exchange systems in the *Aedes aegypti* mosquito and the *Plutella xylostella* moth. *PLoS ONE* 10, e0121097. doi: 10.1371/journal.pone.0121097

Halder, G., Callaerts, P. and Gehring, W.J. (1995) Induction of ectopic eyes by targeted expression of the eyeless gene in *Drosophila*. *Science* 267, 1788–1792. doi: 10.1126/science.7892602

Handler, A.M., Zimowska, G.J. and Horn, C. (2004) Post-integration stabilization of a transposon vector by terminal sequence deletion in *Drosophila melanogaster*. *Nature Biotechnology* 22, 1150–1154. doi: 10.1038/nbt1002

He, B., Buescher, M., Farnworth, M.S., Strobl, F., Stelzer, E.H. *et al.* (2019) An ancestral apical brain region contributes to the central complex under the control of foxQ2 in the beetle *Tribolium*. *eLife* 8, e49065. doi: 10.7554/eLife.49065

He, Z., Proudfoot, C., Mileham, A.J., McLaren, D.G., Whitelaw, C.B.A. and Lillico, S.G. (2015) Highly efficient targeted chromosome deletions using CRISPR/Cas9. *Biotechnology and Bioengineering* 112, 1060–1064. doi: 10.1002/bit.25490

Heigwer, F., Port, F. and Boutros, M. (2018) RNA Interference (RNAi) screening in *Drosophila*. *Genetics* 208, 853–874. doi: 10.1534/genetics.117.300077

Heinrich, J.C. and Scott, M.J. (2000) A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. *Proceedings of the National Academy of Sciences USA* 97, 8229–8232. doi: 10.1073/pnas.140142697

Henikoff, S. (1992) Position effect and related phenomena. *Current Opinion in Genetics & Development* 2, 907–912. doi: 10.1016/S0959-437X(05)80114-5

Horn, C. and Handler, A.M. (2005) Site-specific genomic targeting in *Drosophila*. *Proceedings of the National Academy of Sciences USA* 102, 12483–12488. doi: 10.1073/pnas.0504305102

Horn, C. and Wimmer, E.A. (2003) A transgene-based, embryo-specific lethality system for insect pest management. *Nature Biotechnology* 21, 64–70. doi: 10.1038/nbt769

Huang, J., Zhou, W., Dong, W., Watson, A.M. and Hong, Y. (2009) Directed, efficient, and versatile modifications of the *Drosophila* genome by genomic engineering. *Proceedings of the National Academy of Sciences USA* 106, 8284–8289. doi: 10.1073/pnas.0900641106

Huang, J., Ghosh, P., Hatfull, G.F. and Hong, Y. (2011) Successive and targeted DNA integrations in the *Drosophila* genome by *Bxb1* and $\phi C31$ integrases. *Genetics* 189, 391–395. doi: 10.1534/genetics.111.129247

Huet, F., Lu, J.T., Myrick, K.V., Baugh, L.R., Crosby, M.A. and Gelbart, W.M. (2002) A deletion-generator compound element allows deletion saturation analysis for genomewide phenotypic annotation. *Proceedings of the National Academy of Sciences USA* 99, 9948–9953. doi: 10.1073/pnas.142310099

Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q. *et al.* (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature Biotechnology* 31, 227–229. doi: 10.1038/nbt.2501

Ishibashi, K., Suzuki, K., Ando, Y., Takakura, C. and Inoue, H. (2006) Nonhomologous chromosomal integration of foreign DNA is completely dependent on MUS-53 (human Lig4 homolog) in *Neurospora*. *Proceedings of the National Academy of Sciences USA* 103, 14871–14876. doi: 10.1073/pnas.0604477103

Iwata, S., Yoshina, S., Suehiro, Y., Hori, S. and Mitani, S. (2016) Engineering new balancer chromosomes in *C. elegans* via CRISPR/Cas9. *Scientific Reports* 6, 33840. doi: 10.1038/srep33840

Jasin, M. (1996) Genetic manipulation of genomes with rare-cutting endonucleases. *Trends in Genetics* 12, 224–228. doi:.1016/0168-9525(96)10019-6

Jasinskiene, N. (2003) High efficiency, site-specific excision of a marker gene by the phage *P1* cre-loxP system in the yellow fever mosquito, *Aedes aegypti*. *Nucleic Acids Research* 31, 147e–1147. doi: 10.1093/nar/gng148

Jiang, J., Zhang, L., Zhou, X., Chen, X., Huang, G. *et al.* (2016) Induction of site-specific chromosomal translocations in embryonic stem cells by CRISPR/Cas9. *Scientific Reports* 6, 21918. doi: 10.1038/srep21918

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821. doi: 10.1126/science.1225829

Kanca, O., Zirin, J., Garcia-Marques, J., Knight, S.M., Yang-Zhou, D. *et al.* (2019) An efficient CRISPR-based strategy to insert small and large fragments of DNA using short homology arms. *eLife* 8, e51539. doi: 10.7554/eLife.51539

Kandul, N.P., Belikoff, E.J., Liu, J., Buchman, A., Li, F. *et al.* (2021) Genetically encoded CRISPR components yield efficient gene editing in the invasive pest, *Drosophila suzukii*. *CRISPR Journal* 4, 739–751. doi: 10.1089/crispr.2021.0032

Carrami, E.M., Eckermann, K.N., Ahmed, H.M.M., Sánchez C., H.M., Dippel, S. *et al.* (2018) Consequences of resistance evolution in a Cas9-based sex conversion-suppression gene drive for insect pest management. *Proceedings of the National Academy of Sciences USA* 115, 6189–6194. doi: 10.1073/pnas.1713825115

Kaufmann, B.P. (1939) Induced chromosome rearrangements in *Drosophila melanogaster*. *Journal of Heredity* 30, 179–190. doi: 10.1093/oxfordjournals.jhered.a104712

Kosman, D. and Small, S. (1997) Concentration-dependent patterning by an ectopic expression domain of the *Drosophila* gap gene *knirps*. *Development* 124, 1343–1354.

Labbé, G.M.C., Nimmo, D.D. and Alphey, L. (2010) *piggyBac*- and *PhiC31*-mediated genetic transformation of the Asian tiger mosquito, *Aedes albopictus* (Skuse). *PLoS Neglected Tropical Diseases* 4, e788. doi: 10.1371/journal.pntd.0000788

Landy, A. (1989) Dynamic, structural, and regulatory aspects of lambda site-specific recombination. *Annual Review of Biochemistry* 58, 913–949. doi: 10.1146/annurev.bi.58.070189.004405

Lee, Y.S. and Carthew, R.W. (2003) Making a better RNAi vector for *Drosophila*: use of intron spacers. *Methods* 30, 322–329. doi: 10.1016/s1046-2023(03)00051-3

Li, J. and Handler, A.M. (2017) Temperature-dependent sex-reversal by a transformer-2 gene-edited mutation in the spotted wing drosophila, *Drosophila suzukii*. *Scientific Reports* 7, 12363. doi: 10.1038/s41598-017-12405-4

Li, K., Wang, G., Andersen, T., Zhou, P. and Pu, W.T. (2014) Optimization of genome engineering approaches with the CRISPR/Cas9 system. *PLoS ONE* 9, e105779. doi: 10.1371/journal.pone.0105779

Liang, F., Han, M., Romanienko, P.J. and Jasin, M. (1998) Homology-directed repair is a major double-strand break repair pathway in mammalian cells. *Proceedings of the National Academy of Sciences USA* 95, 5172–5177. doi: 10.1073/pnas.95.9.5172

Long, D., Lu, W., Zhang, Y., Bi, L., Xiang, Z. and Zhao, A. (2015) An efficient strategy for producing a stable, replaceable, highly efficient transgene expression system in silkworm, *Bombyx mori*. *Scientific Reports* 5, 8802. doi: 10.1038/srep08802

Long, D.-P., Zhao, A.-C., Chen, X.-J., Zhang, Y., Lu, W.-J. *et al.* (2012) FLP recombinase-mediated site-specific recombination in silkworm, *Bombyx mori*. *PLoS ONE* 7, e40150. doi: 10.1371/journal.pone.0040150

Ma, S., Chang, J., Wang, X., Liu, Y., Zhang, J. *et al.* (2014) CRISPR/Cas9 mediated multiplex genome editing and heritable mutagenesis of *BmKu70* in *Bombyx mori*. *Scientific Reports* 4, 4489. doi: 10.1038/srep04489

Makarova, K.S., Grishin, N.V., Shabalina, S.A., Wolf, Y.I. and Koonin, E.V. (2006) A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. *Biology Direct* 1, 7. doi: 10.1186/1745-6150-1-7

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M. *et al.* (2013) RNA-guided human genome engineering via Cas9. *Science* 339, 823–826. doi: 10.1126/science.1232033

Maresca, M., Lin, V.G., Guo, N. and Yang, Y. (2013) Obligate Ligation-Gated Recombination (ObLiGaRe): Custom-designed nuclease-mediated targeted integration through nonhomologous end joining. *Genome Research* 23, 539–546. doi: 10.1101/gr.145441.112

McVey, M. and Lee, S.E. (2008) MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. *Trends in Genetics* 24, 529–538. doi: 10.1016/j.tig.2008.08.007

Meredith, J.M., Basu, S., Nimmo, D.D., Larget-Thiery, I., Warr, E.L. *et al.* (2011) Site-specific integration and expression of an anti-malarial gene in transgenic *Anopheles gambiae* significantly reduces plasmodium infections. *PLoS ONE* 6, e14587. doi: 10.1371/journal.pone.0014587

Meredith, J.M., Underhill, A., McArthur, C.C. and Eggleston, P. (2013) Next-generation site-directed transgenesis in the malaria vector mosquito *Anopheles gambiae*: self-docking strains expressing germline-specific *phiC31* integrase. *PLoS ONE* 8, e59264. doi: 10.1371/journal.pone.0059264

Miller, J.C., Tan, S., Qiao, G., Barlow, K.A., Wang, J. *et al.* (2011) A TALE nuclease architecture for efficient genome editing. *Nature Biotechnology* 29, 143–148. doi: 10.1038/nbt.1755

Moynahan, M.E. and Jasin, M. (2010) Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. *Nature Reviews Molecular Cell Biology* 11, 196–207. doi: 10.1038/nrm2851

Nash, H.A. and Robertson, C.A. (1981) Purification and properties of the *Escherichia coli* protein factor required for *lambda* integrative recombination. *Journal of Biological Chemistry* 256, 9246–9253.

Nimmo, D.D., Alphey, L., Meredith, J.M. and Eggleston, P. (2006) High efficiency site-specific genetic engineering of the mosquito genome. *Insect Molecular Biology* 15, 129–136. doi: 10.1111/j.1365-2583.2006.00615.x

Nussenzweig, A. and Nussenzweig, M.C. (2007) A backup DNA repair pathway moves to the forefront. *Cell* 131, 223–225. doi: 10.1016/j.cell.2007.10.005

Oberstein, A., Pare, A., Kaplan, L. and Small, S. (2005) Site-specific transgenesis by Cre-mediated recombination in *Drosophila*. *Nature Methods* 2, 583–585. doi: 10.1038/nmeth775

O'Brochta, D.A. and Handler, A.M. (1988) Mobility of P elements in drosophilids and nondrosophilids. *Proceedings of the National Academy of Sciences USA* 85, 6052–6056. doi: 10.1073/pnas.85.16.6052

Parks, A.R. and Peters, J.E. (2018) Conservative site-specific recombination. In: Wells, R.D., Bond, J.S., Klinman, J. and Masters, B.S.S. (eds) *Molecular Life Sciences*. Springer New York, New York, pp. 119–127. doi: 10.1007/978-1-4614-1531-2_165

Perrimon, N. and Gans, M. (1983) Clonal analysis of the tissue specificity of recessive female-sterile mutations of *Drosophila melanogaster* using a dominant female-sterile mutation Fs(1)K1237. *Developmental Biology* 100, 365–373. doi: 10.1016/0012-1606(83)90231-2

Platt, R.J., Chen, S., Zhou, Y., Yim, M.J., Swiech, L. *et al.* (2014) CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell* 159, 440–455. doi: 10.1016/j.cell.2014.09.014

Pondeville, E., Puchot, N., Meredith, J.M., Lynd, A., Vernick, K.D. *et al.* (2014) Efficient *ΦC31* integrase–mediated site-specific germline transformation of *Anopheles gambiae*. *Nature Protocols* 9, 1698–1712. doi: 10.1038/nprot.2014.117

Port, F., Chen, H.-M., Lee, T. and Bullock, S.L. (2014) Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in *Drosophila*. *Proceedings of the National Academy of Sciences USA* 111, E2967–E2976. doi: 10.1073/pnas.1405500111

Preston, R.J. (2014) Chromosome aberrations. In: Wexler, P. (ed.) *Encyclopedia of Toxicology* (3rd edn). Academic Press, Oxford, pp. 955–958. doi: 10.1016/B978-0-12-386454-3.00010-5

Preston, C.R., Sved, J.A. and Engels, W.R. (1996) Flanking duplications and deletions associated with *P*-induced male recombination in *Drosophila*. *Genetics* 144, 1623–1638. doi: 10.1093/genetics/144.4.1623

Rausch, H. and Lehmann, M. (1991) Structural analysis of the actinophae *ΦC31* attachment site. *Nucleic Acids Research* 19, 5187–5189. doi: 10.1093/nar/19.19.5187

Reid, W. and O'Brochta, D.A. (2016) Applications of genome editing in insects. *Current Opinion in Insect Science* 13, 43–54. doi: 10.1016/j.cois.2015.11.001

Rio, D.C. and Rubin, G.M. (1988) Identification and purification of a *Drosophila* protein that binds to the terminal 31-base-pair inverted repeats of the P transposable element. *Proceedings of the National Academy of Sciences USA* 85, 8929–8933. doi: 10.1073/pnas.85.23.8929

Robinson, A.S. and Van Heemert, C. (1982) *Ceratitis capitata*—a suitable case for genetic sexing. *Genetica* 58, 229–237. doi: 10.1007/BF00128017

Rong, Y.S. and Golic, K.G. (2000) Gene targeting by homologous recombination in *Drosophila*. *Science* 288, 2013–2018. doi: 10.1126/science.288.5473.2013

Rouet, P., Smih, F. and Jasin, M. (1994) Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Molecular and Cellular Biology* 14, 11.

Rubin, G.M. and Spradling, A.C. (1982) Genetic transformation of *Drosophila* with transposable element vectors. *Science* 218, 348–353. doi: 10.1126/science.6289436

Rutherford, K., Yuan, P., Perry, K., Sharp, R. and Van Duyne, G.D. (2013) Attachment site recognition and regulation of directionality by the serine integrases. *Nucleic Acids Research* 41, 8341–8356. doi: 10.1093/nar/gkt580

Ryder, E., Blows, F., Ashburner, M., Bautista-Llacer, R., Coulson, D. *et al.* (2004) The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in *Drosophila melanogaster*. *Genetics* 167, 797–813. doi: 10.1534/genetics.104.026658

Ryder, E., Ashburner, M., Bautista-Llacer, R., Drummond, J., Webster, J. *et al.* (2007) The DrosDel Deletion Collection: a *Drosophila* genomewide chromosomal deficiency resource. *Genetics* 177, 615–629. doi: 10.1534/genetics.107.076216

Schetelig, M.F., Scolari, F., Handler, A.M., Kittelmann, S., Gasperi, G. and Wimmer, E.A. (2009) Site-specific recombination for the modification of transgenic strains of the Mediterranean fruit fly *Ceratitis capitata*. *Proceedings of the National Academy of Sciences USA* 106, 18171–18176. doi: 10.1073/pnas.0907264106

Schetelig, M.F., Götschel, F., Viktorinová, I., Handler, A.M. and Wimmer, E.A. (2011) Recombination technologies for enhanced transgene stability in bioengineered insects. *Genetica* 139, 71–78. doi: 10.1007/s10709-010-9494-4

Schetelig, M.F., Yan, Y., Zhao, Y. and Handler, A.M. (2019) Genomic targeting by recombinase-mediated cassette exchange in the spotted wing drosophila, *Drosophila suzukii*. *Insect Molecular Biology* 28, 187–195. doi: 10.1111/imb.12537

Schmitt-Engel, C., Schultheis, D., Schwirz, J., Ströhlein, N., Troelenberg, N. *et al.* (2015) The iBeetle large-scale RNAi screen reveals gene functions for insect development and physiology. *Nature Communications* 6, 7822. doi: 10.1038/ncomms8822

Shimomura, O., Johnson, F.H. and Saiga, Y. (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *Journal of Cellular and Comparative Physiology* 59, 223–239. doi: 10.1002/jcp.1030590302

Siegal, M.L. and Hartl, D.L. (1996) Transgene coplacement and high efficiency site-specific recombination with the Cre/loxP system in *Drosophila*. *Genetics* 144, 715–726. doi: 10.1093/genetics/144.2.715

Skipper, K.A., Andersen, P.R., Sharma, N. and Mikkelsen, J.G. (2013) DNA transposon-based gene vehicles – scenes from an evolutionary drive. *Journal of Biomedical Sciences* 20, 92. doi: 10.1186/1423-0127-20-92

Spradling, A.C. and Rubin, G.M. (1982) Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science* 218, 341–347. doi: 10.1126/science.6289435

Spradling, A.C. and Rubin, G.M. (1983) The effect of chromosomal position on the expression of the *Drosophila* xanthine dehydrogenase gene. *Cell* 34, 47–57. doi: 10.1016/0092-8674(83)90135-6

Sternberg, N. and Hamilton, D. (1981) Bacteriophage *P1* site-specific recombination. I. Recombination between *loxP* sites. *Journal of Molecular Biology* 150, 467–486. doi: 10.1016/0022-2836(81)90375-2

Struhl, G. and Basler, K. (1993) Organizing activity of *wingless* protein in *Drosophila*. *Cell* 72, 527–540. doi: 10.1016/0092-8674(93)90072-X

Thacker, D. and Keeney, S. (2016) Homologous recombination during meiosis. In: Hanaoka, F. abd Sugasawa, K. (eds) *DNA Replication, Recombination, and Repair*. Springer Japan, Tokyo, pp. 131–151. doi: 10.1007/978-4-431-55873-6_6

Thorpe, H.M., Wilson, S.E. and Smith, M.C.M. (2000) Control of directionality in the site-specific recombination system of the *Streptomyces* phage $\phi C31$. *Molecular Microbiology* 38, 232–241. doi: 10.1046/j.1365-2958.2000.02142.x

Tkachuk, A., Kim, M., Kravchuk, O. and Savitsky, M. (2011) A new powerful method for site-specific transgene stabilization based on chromosomal double-strand break repair. *PLoS ONE* 6, e26422. doi: 10.1371/journal.pone.0026422

Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S. and Gregory, P.D. (2010) Genome editing with engineered zinc finger nucleases. *Nature Reviews Genetics* 11, 636–646. doi: 10.1038/nrg2842

Vaidya, A., Mao, Z., Tian, X., Spencer, B., Seluanov, A. and Gorbunova, V. (2014) Knock-in reporter mice demonstrate that DNA repair by non-homologous end joining declines with age. *PLoS Genetics* 10, e1004511. doi: 10.1371/journal.pgen.1004511

Venken, K.J.T. and Bellen, H.J. (2005) Emerging technologies for gene manipulation in *Drosophila melanogaster*. *Nature Reviews Genetics* 6, 167–178. doi: 10.1038/nrg1553

Venken, K.J.T., He, Y., Hoskins, R.A. and Bellen, H.J. (2006) P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in *D. melanogaster*. *Science* 314, 1747–1751. doi: 10.1126/science.1134426

Wang, B., Li, K., Wang, A., Reiser, M., Saunders, T. *et al.* (2015) Highly efficient CRISPR/HDR-mediated knock-in for mouse embryonic stem cells and zygotes. *Biotechniques* 59, 201–202, 204, 206–208. doi: 10.2144/000114339

Wang, F., Ji, Y.T., Tian, C., Wang, Y.C., Xu, S. *et al.* (2021) An inducible constitutive expression system in *Bombyx mori* mediated by *phiC31* integrase. *Insect Science* 28, 1277–1289. doi: 10.1111/1744-7917.12866.

Ward, C.M., Aumann, R.A., Whitehead, M.A., Nikolouli, K., Leveque, G. *et al.* (2021) White pupae phenotype of tephritids is caused by parallel mutations of a MFS transporter. *Nature Communications* 12**,** 491.

Wieschaus, E., Audit, C. and Masson, M. (1981) A clonal analysis of the roles of somatic cells and germ line during oogenesis in *Drosophila*. *Developmental Biology* 88, 92–103. doi: 10.1016/0012-1606(81)90221-9.

Wimmer, E.A. (2005) Insect transgenesis by site-specific recombination. *Nature Methods* 2, 580–582. doi: 10.1038/nmeth0805-580

Wimmer, E.A., Cohen, S.M., Jäckle, H. and Desplan, C. (1997) *buttonhead* does not contribute to a combinatorial code proposed for *Drosophila* head development. *Development* 124, 1509–1517.

Xu, T. and Rubin, G. (1993) Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* 117, 1223–1237.

Yan, Y., Kobayashi, Y., Huang, C., Liu, B., Qian, W. *et al.* (2021) Highly efficient temperature inducible CRISPR-Cas9 gene targeting in *Drosophila suzukii*. *International Journal of Molecular Sciences* 22, 6724.

Zhu, L., Mon, H., Xu, J., Lee, J.M. and Kusakabe, T. (2015) CRISPR/Cas9-mediated knockout of factors in non-homologous end joining pathway enhances gene targeting in silkworm cells. *Scientific Reports* 5, 18103. doi: 10.1038/srep18103

6   Receptor-Mediated Ovary Transduction of Cargo – ReMOT Control: a Comprehensive Review and Detailed Protocol for Implementation

Gerard Terradas, Vanessa M. Macias, Hillary Peterson, Sage McKeand, Grzegorz Krawczyk and Jason L. Rasgon*

Department of Entomology, the Center for Infectious Disease Dynamics, and the Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, Pennsylvania, USA

6.1   History of Transgenic Methods in Arthropods

All biological fields have been impacted by technological advances in the past 25 years. None has developed faster than DNA sequencing and bioinformatics, leading to a deep understanding of the biology and genetics of non-model organisms relevant to public health, agriculture and socio-economics. Currently, full genome annotations of more than 150 insects have been publicly released (mostly Diptera and Hymenoptera) (Li *et al*., 2019), as well as extensive population data to account for field diversity in an effort to control malaria (1000 Genomes Consortium) (Miles *et al*., 2017). While there have been great advances in insect vector genomics and the field is continuously developing, the ability to easily obtain and retrieve sequencing and 'omics' data allows for the design of targeted gene manipulation of organisms and enables further exploration of gene function, genetic networks and interactions between vectors and the pathogens they transmit.

Genetic manipulation of arthropods of medical and economic importance has always been and continues to be a challenge in the entomological field. Classic vector genome modification techniques (Table 6.1) date back to the 1990s, when viral or plasmid expression vectors (baculovirus: Maeda *et al*., 1985; SINV: Higgs *et al*., 1995; plasmid: Cornel *et al*., 1997) and transposon-mediated integration (*P-element*: Miller *et al*., 1987; *Hermes*: Jasinskiene *et al*., 1998; *Minos*: Catteruccia *et al*., 2000; *piggyBac*: Kokoza *et al*., 2001) were used to transiently or stably express foreign DNA in mosquitoes to study their biology, biocontrol and production of non-native gene products. The delivery of DNA to germ cells enables the creation of heritable genetic changes that are transmitted through generations. Traditionally, the options to create such modified arthropod lines have relied on direct microinjection of early-stage embryos with gene vectors that produce a random (*piggyBac*, *Hermes*, *Minos*, *Mos1*) or site-specific (φC31, TALEN, zinc-finger nucleases) insertions

---

*Corresponding author, email: jlr54@psu.edu

Table 6.1. Comparison between classic and newly-developed gene-editing technologies. In blue, technologies that are currently being used and developed in basic or applied entomological research.

	Transposons	φC31	Homing Endonuclease Gene	Zinc-Finger Nuclease	TALEN	CRISPR/Cas9		
						Embryo mutagenesis	Gene drive	ReMOT Control
Biological origin	DNA type-II transposable elements discovered in maize	Phage DNA integration into bacterial genomes via φC31 recombinase	Natural selfish genetic elements capable to be transmitted horizontally in Super-Mendelian fashion	Fusion of a zinc-binding domain shared among transcription factors that recognize DNA with a FokI endonuclease to mediate site-specific cleavage	TALE proteins from *Xanthomonas* conjugated to FokI endonuclease	Prokaryotic defence system acting as acquired immunity by storing DNA sequences of previously-infecting phages. Upon recognition of an incoming phage containing a stored target sequence, Cas9 eliminates the pathogen by DNA cleavage		
First use in genome editing	1982 (*D. melanogaster*)	1982 (*S. pyogenes*)	1998 (*E. coli*)	2001 (*X. laevis*)	2010 (*S. cerevisiae*)	2013 (human cells)		
First use in mosquitoes	1998 (*Ae. aegypti*)	2006 (*Ae. aegypti*)	2011 (*An. gambiae*)	2013 (*Ae. aegypti*)	2013 (*An. gambiae*)	2015 (*Ae. aegypti*)	2015 (*An. stephensi*)	2018 (*Ae. aegypti*)
Mode of action	Transfer of DNA located between inverted terminal repeats from a DNA source (plasmid) into a random location in the target genome	Site-specific recombination occurs between AttB (donor) and AttP (target) sequences	I-SceI (HEG) recognizes and cleaves DNA for a gene cassette to integrate into the cutsite via HDR	ZFN/TALE domains recognize a sequence of nucleotide triplets, a pair of endonucleases create a DNA double-stranded break		Cas9-sgRNA RNP complexes mediate recognition and cleavage of a specific genomic DNA location. NHEJ repair creates knockout mutations	Upon Cas9 cleavage, the chromosome is repaired using the drive-containing homologous chromosome (or template DNA). This process leads to an autonomously spreading genetic element *in vivo*	P2C (or other ovary-targeting ligand) leads Cas9-sgRNA RNP complexes to the adult ovaries for Cas9 cleavage to occur in the growing oocytes, editing the germline

Efficiency	2-3%	10-20%	<1%	<6%	2%	2–3%	1–2%	1–6%
Knock-in	Yes	Yes	Yes	Yes	Yes	No	Yes	No
Drive capacity	Some	No	Yes	No	No	No	Yes	No
Drive efficiency	Low	–	60%	–	–	–	90–100%	–
Pros	First available technique for insects. Lots of resources widely available	Site-specific and high transformation efficiency. Resources constitutively expressing φC31 recombinase	Site-specific, drive capacity	Site-specific	Site-specific, efficient mutagenesis and TALENs can be produced relatively easily	Site-specific, adaptable to many species, easy to target multiple genes. Great efficiency and versatility.		
						Easy to re-engineer and adapt to many gene targets	Self-propagating genetic element, easy to produce a homozygous mutant stock of any GOI. Capacity to alter wild populations	Adult injections are easier and less difficult to perform. Cheap, does not require specialized equipment
Cons	Random insertion requiring molecular procedures to detect insertion site, relatively low efficiency	Requires a docking line with pre-inserted AttP sequence	Drive-resistant alleles, requires pre-existing target sites and laborious re-engineering of the HEG to target different genomic locations	Requires modular protein engineering and *in vitro* optimization, zinc-finger recognition may be difficult for some codons	Requires protein engineering and timing: the versatile CRISPR/Cas9 took the spotlight	Potential off-target effects, mutants difficult to capture without visible phenotypes	Resistant alleles	No HDR to induce knock-ins, mutants difficult to capture without visible phenotypes

into the genome (Table 6.1) (see O'Brochta, Chapter 1; Ahmed and Wimmer, Chapter 5, this volume). The most commonly used systems in insect transgenesis are *piggyBac* transposon (Handler and Harrell, 1999; Grossman *et al.*, 2001; Kokoza *et al.*, 2001) and φC31 (Nimmo *et al.*, 2006; Labbé *et al.*, 2010), which have proven successful for a range of species and for which reagents (such as insect lines that constitutively express PB transposase, φC31 recombinase, or contain AttP/AttB docking sites) are widely available. Other more straightforward alternatives such as biolistics (Kravariti *et al.*, 2001) or electroporation (Thomas, 2003) that were developed early on for manipulation in somatic tissues are showing promise for germline transformation (Jamison *et al.*, 2018; Lule-Chávez *et al.*, 2021).

6.2 Development of CRISPR-based Technologies

Following the discovery and subsequent impact in medicine and biotechnology of CRISPR/Cas technologies (Jinek *et al.*, 2012) (Table 6.1), the transgenesis field changed completely. In nature, CRISPR/Cas acts as an adaptive heritable immune system in prokaryotes, providing protection from viral DNA and plasmids (Mojica and Rodriguez-Valera, 2016). However, the system was redesigned with the fusion of the tracrRNA and crRNA into a single adaptable guide RNA (sgRNA) (Jinek *et al.*, 2012) and then modified for *in vitro* and *in vivo* genome editing of eukaryotic cells and organisms (Cong *et al.*, 2013; Gratz *et al.*, 2013; Jinek *et al.*, 2013). The typical CRISPR/Cas system relies on the DNA nuclease spCas9 (or CRISPR-associated protein 9 from *Streptococcus pyogenes*) and a site-specific sgRNA that can be tailored to target any genomic sequence positioned next to a protospacer adjacent motif (PAM) (see Concha and Papa, Chapter 7, this volume). Cas9 and sgRNA bind, forming a negatively charged ribonucleic protein complex with the sgRNA guiding Cas9 to the DNA cut site. In an *in vivo* genetic modification context, the ability to cleave the DNA at

a precise genomic location allows for targeted mutagenesis of any gene within an organism's genome. When a CRISPR-induced DNA double-stranded break occurs, the organism repairs it mostly via one of the following two mutually exclusive processes: (i) homology-directed repair (HDR), which uses the homologous chromosome or other homologous sequence as a template to copy the missing sequence into the break; or (ii) by the non-homologous end joining (NHEJ) pathway, which occurs faster (hence more often) and results in indels in the sequence that can create loss-of-function mutations. This duality in DNA repair mechanisms allowed CRISPR to be used efficiently to generate gene knockouts via NHEJ (Gratz *et al.*, 2013; Wang *et al.*, 2013; Dong *et al.*, 2015; Gilles *et al.*, 2015) as well as knock-ins via HDR (Gratz *et al.*, 2013; Gantz *et al.*, 2015; Kistler *et al.*, 2015). By using a donor DNA template (as ssDNA, dsDNA or plasmid) that has flanking sequences to a specific cut site, the cellular machinery is tricked into processing it as if it were the homologous chromosome. Researchers can manipulate which sequences are added in between these flanking regions with no limitations except cargo size (cargoes bigger than 20 kb in length do not transform well). This way, Cas9 can create a natural knock-in hotspot in any part of the genome and the system can be used to introduce any desired sequence by modifying only two elements: the sgRNA target sequence and the DNA template. CRISPR technologies also have the potential to activate or repress *in vivo* gene expression. A mutant form of Cas9 (dCas9), which does not possess endonuclease activity, was developed to study the catalytic domains needed for Cas9 cleavage (Jinek *et al.*, 2012), but rapidly became a DNA-binding tool for transcriptional activation or repression (Bikard *et al.*, 2013; Mali *et al.*, 2013; Qi *et al.*, 2013), gene expression modification (Cheng *et al.*, 2013; Gilbert *et al.*, 2013) and editing of epigenetic factors (Hilton *et al.*, 2015).

The use of Cas9 and associated CRISPR technologies revolutionized not only the study of insect biology but also the population control field, particularly in those species that cause public health outbreaks (*Aedes*,

Anopheles, *Culex*) or losses of agricultural crops (*Bactrocera tabaci*, *Drosophila suzukii*). Traditionally, vector control efforts relied on the use of insecticide-treated nets and pesticides. While they do work quite effectively in areas of high vector and disease prevalence, there is concern about their use due to the environmental damage caused by such chemical compounds and the effects from the appearance of insecticide-resistant strains. Although the use of selfish elements to replace wild populations had been theorized for some time (Burt, 2003), the surge of CRISPR/Cas9 technologies, along with the capacity to genetically encode its components, allowed for the creation of the first practical autonomous gene drive in insects (Gantz and Bier, 2015) (Table 6.1). Most synthetic gene drives currently in development are genetically encoded Cas9-based selfish elements that have the capacity to self-spread by biasing their inheritance from Mendelian (50%) to super-Mendelian (> 50%) and can be adapted to either crash ('suppression') or introduce beneficial traits ('modification') into a wild population (see Raban and Akbari, Chapter 8; Champer, Chapter 9; Bottino-Rojas and James, Chapter 11, this volume). When Cas9 and an sgRNA are encoded in the same organism, either linked in the same genomic location (Gantz *et al.*, 2015; Hammond *et al.*, 2016; Kyrou *et al.*; 2018, Adolfi *et al.*; 2020, Carballar-Lejarazú *et al.*, 2020) or split in two independent loci (Li *et al.*, 2020; López Del Amo *et al.*, 2020; Terradas *et al.*, 2021), Cas9 cuts the sgRNA target site in the non-drive allele of the germline. That is where HDR occurs and the non-drive chromosome is repaired using the homologous drive allele as a donor, thus biasing the inheritance of the drive towards super-Mendelian frequencies. In the case of population modification, the spread of the drive allele will be linked to the spread of a beneficial cargo (for example, a pathogen-refractory effector (Isaacs *et al.*, 2011; Dong *et al.*, 2020)). For population suppression, the spread of the drive will carry a detrimental trait that hampers their survival either via suppression of female fertility (Hammond *et al.*, 2016), sex-ratio distortion by genetic selection of male offspring (Kyrou *et al.*, 2018) or targeted destruction of the X chromosome (Galizi *et al.*, 2014; Simoni *et al.*, 2020) (see Arien *et al.*, Chapter 10, this volume). While, to date, the effectiveness of gene drives has not been assessed in field trials, the results from laboratory-caged experiments offer high potential for the technology (Hammond *et al.*, 2021; Adolfi *et al.*, 2020).

6.3 Problems with Traditional Embryonic Microinjection

The genome manipulation technologies described above form the current state of molecular genetic arthropod control strategies. A bottleneck of these techniques is their need to deliver exogenous substances, such as donor DNA constructs, proteins, or peptides specific to each transgenesis system (i.e., transposase, recombinase, Cas9), at embryonic stages in a time- and location-specific manner. Microinjection offers the capacity to incorporate precise minuscule amounts of these compounds into insect eggs with relatively high efficiency, compared with other chemical (endocytosis (Colosimo *et al.*, 2000)) or physical (electroporation (Thomas, 2003), gene gun (Kravariti *et al.*, 2001)) manipulation techniques. However, insect egg micromanipulation and injection have significant limitations, as the target embryo receives physical damage that potentially reduces its viability and development. Transgenes are introduced into the pre-blastocyst posterior pole of the embryos (Jasinskiene *et al.*, 2007) and efficiency is dependent on injection volume and pressure, desiccation, buffer pH and compatibility of the exogenous substance to the target insect. Reproducibility can be hard to achieve due to user-dependent technique, variable purities of different injection mixes or temperature and humidity of the room at the time of injection, which alters desiccation time. All of these factors can lead to experimental failure. Additionally, while microinjection methods are able to accommodate unique characteristics (mainly egg shape and developmental time) of each insect, many non-model species are not adaptable to micromanipulation or injection, because their eggs are too damaged during

the procedure, they do not have the capacity to lay enough synchronous eggs (burying beetle (Smiseth *et al.*, 2006)), they give live birth rather than ovipositing (tsetse fly (Benoit *et al.*, 2015)) or the eggs require anchoring to certain host tissues (Asian citrus psyllid (Hall *et al.*, 2013)).

Besides biological and technical challenges, embryonic micromanipulation and microinjection are techniques that require extensive training in the use of expensive equipment as well as insect-specific rearing and husbandry. Thus, the development of more straightforward (less training required), economically friendly (cheaper) and less time-consuming (faster) techniques are necessary for the advancement of gene editing. One of these promising strategies is the recently developed Receptor-Mediated Ovary Transduction of cargo (ReMOT Control) technique for germline mutagenesis following haemocoel injections in adult female insects (Fig. 6.1) (Table 6.1). ReMOT Control uses a natural arthropod-based ovarian delivery system to deliver CRISPR components from the haemolymph to the developing oocytes, resulting in genetically modified offspring while bypassing the need for embryonic microinjection (Fig. 6.1).

6.4 ReMOT Control Development

For years, receptor-mediated endocytosis by specific ligands has been explored as a method to deliver foreign material for therapeutics (Wagner *et al.*, 1994; Qian *et al.*, 2002). One of the most successful examples is the use of transferrin, a blood protein that binds to iron and disperses it throughout the body, as a transporter for toxins (Fitz-Gerald *et al.*, 1983), liposomes (Hege *et al.*, 1989), proteins (Wagner *et al.*, 1994) and DNA (Stavridis and Psallidopoulos, 1982). Research on the role of intracellular controllers of its dynamics, such as different Rab proteins, led transferrin to be viewed as a promising drug delivery vehicle. Transferrin was chemically conjugated to bind the molecular cargo and was able to use its receptor to internalize into cells to be released into

the cell cytoplasm (Widera *et al.*, 2003; Chen *et al.*, 2013). Delivery efficacy is dependent on the ability of membrane-bound vesicles to release the cargo (Hege *et al.*, 1989; Wagner *et al.*, 1994; Widera *et al.*, 2003). This is usually amplified by using chemical membrane destabilizers that induce pore formation, partial solubilization or even disruption of the affected membrane (Fuchs *et al.*, 2013). Efficient endosomal escape can be achieved by cell-penetrating cationic amphiphilic peptides (Huang *et al.*, 2004), non-peptidic substances (amines, cationic polymers (Liang and Lam, 2012)) or other molecules (monensin, saponin (Fuchs *et al.*, 2009) or chloroquine (Wu, 1997)).

Most oviparous animals deliver required protein material into developing ovaries during vitellogenesis, a highly conserved ovarian and egg maturation process. Arthropods synthesize yolk-protein precursors (YPPs) in the fat body, which are then secreted into the haemolymph to be taken up by the ovaries via receptor-mediated endocytosis (RME). During vitellogenesis, YPP ligands bind to receptors that are expressed in the oocyte membrane to be internalized into the developing embryo, where they accumulate in endosomal vesicles and are sorted into yolk granules for nutrient storage. In order to achieve Cas9 delivery into the ovary, a ligand derived from a *Drosophila melanogaster* YPP (DmYP1) was fused to a Cas9 ribonucleoprotein (RNP) complex (a molecular cargo consisting of the union of Cas9 protein and sgRNA) (Chaverra-Rodriguez *et al.*, 2018). Recombinant DmYP1 had previously been shown by immunological methods to be internalized by the *Anopheles gambiae* oocytes after intrathoracic injection (Bownes *et al.*, 2002), so it was hypothesized to be a suitable candidate to deliver molecular cargoes into the tissue. For easier bioengineering applications, a smaller functional region was identified (P2C peptide; NLQQQRQHGKNGNQDYQDQSNEQRKN-QRTSSEEDYSEEVKN), which belongs to the N-terminal portion of DmYP1 (Fig. 6.1). P2C is sufficient to deliver cargo into oocytes and represents a tenfold reduction in the length of the protein (from 439 to 41 amino acids), as well as being application-ready for any
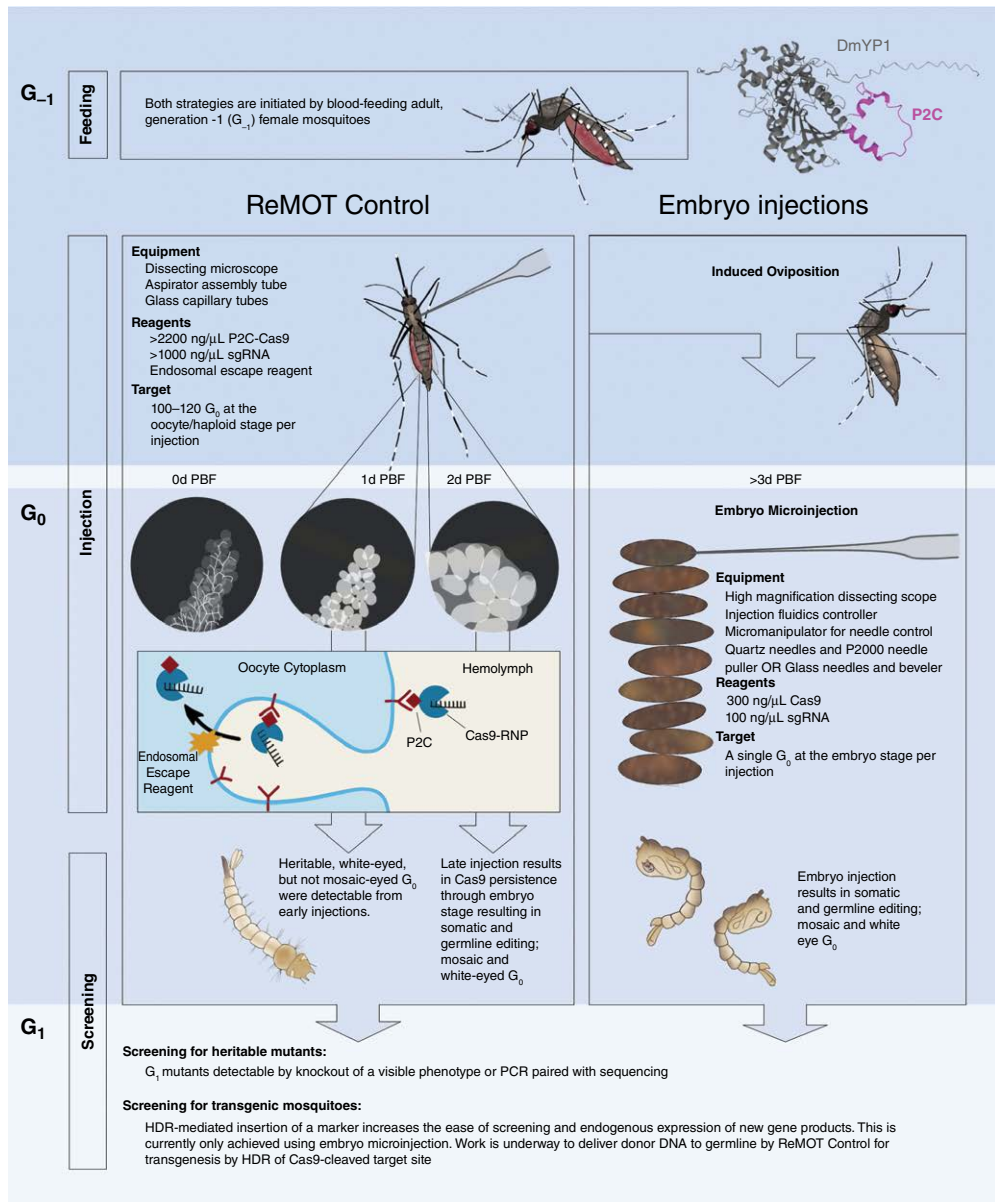
Fig. 6.1. Schematic model of ReMOT Control versus embryonic microinjection in mosquitoes. Both strategies are compared in terms of methodology (injection stage, timing, required equipment and reagents). Top-right corner: structure of DmYP1 (grey), with the P2C domain depicted in pink. Generations (G_x) are indicated at the top-left of each panel and separated using different shades of blue. In G_0, a schematic model of the uptake of P2C-Cas9 RNP into the mosquito oocyte is illustrated.

appropriate arthropod. Upon injection of P2C-Cas9RNP into the thorax of a vitellogenic female, injected P2C-Cas9RNP travels from the haemolymph into the developing oocytes. P2C in the complex leads to high efficiencies of ovarian delivery, which is required to reach the levels of Cas9RNP needed to achieve genome editing in the

embryo. Once the RNP reaches the embryo, it can induce Cas9 cleavage in the sgRNA target site in a similar fashion and efficacy to standard embryonic microinjection-based delivery of CRISPR components (Chaverra-Rodriguez *et al.*, 2018), which produce heritable knockouts by NHEJ indel formation in the genome (Fig. 6.1).

6.5 Summary of ReMOT Control Successes

6.5.1 Mosquitoes

Aedes aegypti – the yellow fever mosquito

The first mosquito used in the development of ReMOT Control was *Aedes aegypti* (Chaverra-Rodriguez *et al.*, 2018). This particular mosquito is the most dangerous animal worldwide, affecting up to 400 million people yearly in more than 200 countries (Leta *et al.*, 2018) due to its high capacity for transmitting several tropical fevers such as dengue, Zika, chikungunya and yellow fever, among others (Souza-Neto *et al.*, 2019). The high impact that *Ae. aegypti* has in public health and socio-economics makes it one of the most studied insect species and it recently became a model for anautogenous species (Matthews and Vosshall, 2020). Mosquitoes also are, in general, excellent specimens to study for technologies based on germline formation, as synchronicity of egg development can be induced by blood feeding and there is substantial literature on vitellogenesis (Raikhel, 1984) and ovarian protein uptake processes (Noah Koller *et al.*, 1989; Attardo *et al.*, 2005). A study used ReMOT Control to knock out the *kynurenine monooxygenase* (*kmo*) gene in *Ae. aegypti* (Han *et al.*, 2003) and demonstrated P2C uptake in *Anopheles* and *Culex* mosquitoes as well, showing the potential adaptability of the technology to multiple species (Chaverra-Rodriguez *et al.*, 2018). As *kmo* has a key role in tryptophan catabolism and synthesis of ommochrome pigments, homozygous recessive mutants produce white-eyed (*kmo*[w]) instead of typical wild-type (*kmo*[+], black-eyed) mosquitoes, facilitating the screening of the null phenotype

from hatching to adulthood. The authors used adult heterozygous mutants for a specific *kmo* base pair position (*kmo*[+]/*kmo*[460]), which display a full black-eyed phenotype, to inject RNP complexes targeting a secondary nucleotide position (519). In the case of successful genome editing, the resulting embryos would display white eyes (*kmo*[w]/*kmo*[w]) as the *kmo*[460] and *kmo*[519] mutations complement. Upon injection of P2C-Cas9 and sgRNA[519] RNP complexes, 1–2% of the hatching G_0 larvae would present a knockout phenotype (Fig. 6.2). This represents an improvement over embryonic microinjections not only in efficiency but also in the number of individuals injected, as the RNPs are introduced to the egg-laying female instead of single embryos.

Anopheles stephensi – Indo-Pakistan malaria vector; and Culex pipiens, the common house mosquito

After this initial proof-of-concept, the technology was applied to other blood-sucking species such as *Anopheles stephensi* (Macias *et al.,* 2020) and *Culex pipiens* (Li *et al.*, 2021). In comparison with *Aedes*, the application of the technology in these species represents a much-needed improvement as they are more recalcitrant to editing and synchronous oviposition and manipulation of the embryos is substantially more difficult to achieve. The study in *C. pipiens* (Li *et al.,* 2021) demonstrated that targeting the *kmo* gene is an approach easily transferable to most other mosquito species and allows for easy quantification of technique efficacy. Injection of sgRNAkmo-bound P2C-Cas9, complemented with either chloroquine or saponin, led to the generation of mosaic and diallelic knockout individuals. Research in *Culex* spp. is challenging, due to the rearing difficulties and obtaining enough injectable eggs from egg rafts, but the ability to use ReMOT Control broadened the gene editing capacities in the species. In *An. stephensi*, the main malaria vector in South(east) Asia and ReMOT's maiden non-*Aedes* arthropod to be edited, Macias *et al.* (2020) knocked out the enhanced cyan fluorescent protein (ECFP) marker from a double-marked line
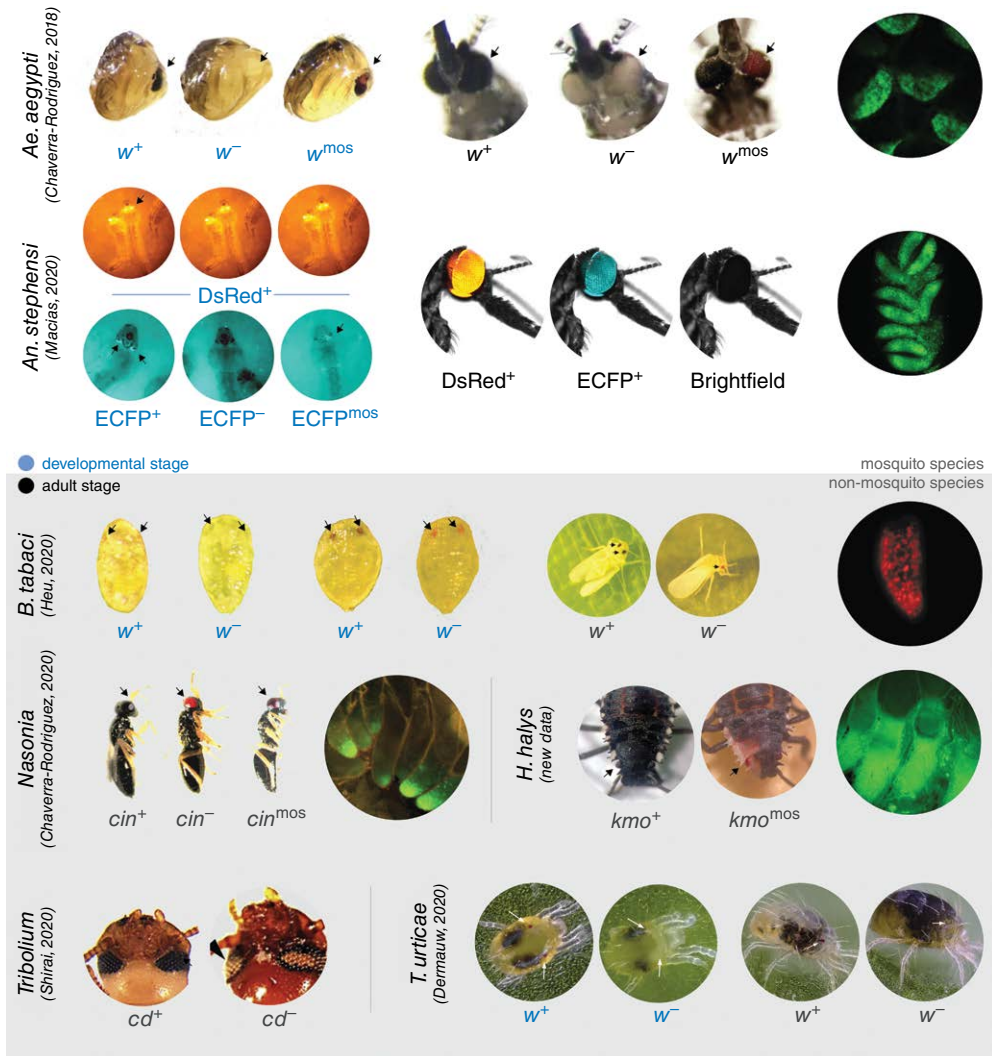
Fig. 6.2. Summary of phenotypes generated using ReMOT Control. Wild-type, knockout and mosaic phenotypes are shown for comparison. In blue, stages that correspond to development; in black, those that belong to adulthood.

(VgCp26.10 (Gantz *et al.*, 2015) – DsRed and ECFP) instead of *kmo*, due to the massive fitness costs that are linked to loss of the gene in the species (Pham *et al.*, 2019) (Fig. 6.2). After injection of RNP complexes with saponin, about 4% of the alleles available for editing resulted in loss of ECFP (Fig. 6.2). This is a high enough percentage to be considered for PCR-based detection of edited alleles in cases where the knockout does not produce a visible phenotype at G_0. As expected, outcrosses of

ECFP G_0 mosquitoes to non-marked wild-type individuals resulted in G_1 Mendelian inheritance of the edited allele.

6.5.2 Non-mosquito insects

Genetic entomological research has traditionally focused on insects that belong to the order Diptera, mainly *Drosophila* and different mosquito species (Chouvenc and Su, 2015).

Nowadays, the development of new genetic tools like CRISPR/Cas technologies have increased the ability to perform biological research in non-model insects. However, CRISPR/Cas needs to be optimized to genetically manipulate certain non-dipteran species with economic and biological value (whiteflies, wasps, beetles, mites). Despite population and evolutionary research being done in those species, the inability to easily create mutant lines has impeded the screening of potential genetic targets that can be used to design agricultural and vector control strategies. All species depicted below share challenges in performing successful microembryonic injection (extreme mortality, embryo size, oviposition, host dependency). ReMOT Control circumvents the need for embryo injection and proves to be a pivotal technical solution to the generation of biological reagents.

Bemisia tabaci – silverleaf whitefly

Bemisia tabaci (cryptic species Middle East-Asia Minor I (MEAM1)), commonly known as silverleaf whitefly, is a polyphagous economically relevant agricultural pest. This insect feeds on phloem sap at all life stages using piercing–sucking mouthparts, which cause direct damage to plants. Its excretions also promote fungus growth that reduces photosynthesis and crop yields, and it is a vector to a range of viruses, e.g. begomoviruses (Czosnek *et al.*, 2017), that affect important crop species. Current control methods for *B. tabaci* are mainly insecticides and predators in greenhouses (Faria and Wraight, 2001; Gerling *et al.*, 2001), so there is a pressing need to develop promising species-specific biocontrol methods. While arthropod gene editing by CRISPR/Cas9 is usually performed by microinjection of editing materials into pre-blastoderm embryos, the exceedingly small size of *B. tabaci* embryos (0.2 mm) and high mortality of injected eggs make it extremely challenging for the species. Thus, whiteflies were a perfect candidate to test ReMOT Control. While the P2C ligand works for multiple mosquito species, it did not work in whiteflies. Instead, ReMOT in whiteflies was developed by using a vitellogenin-based

peptide (BtKV; KPYGVYKTMEDSV) that proved suitable for the species as ovary transducer (Heu *et al.*, 2020). Ovarian development in *B. tabaci* does not resemble that of blood-sucking insects, as it follows an asynchronous pattern with oocytes continuously developing, but endogenous vitellogenin had been shown to be upregulated and endocytosed by phase II oocytes (Guo *et al.*, 2016). The *white* (*w*) gene of *B. tabaci* was targeted with multiple sgRNA in order to test the efficacy of ReMOT in the species. *white* encodes for an ABC transporter protein responsible for ommochrome pigment transport into the eyes and its null mutants display a white-eyed phenotype in most insects (Morgan, 1910; Chaverra-Rodriguez *et al.*, 2018; Feng *et al.*, 2021). Mutants were recovered from 7/9 experiments (Fig. 6.2) when adult females < 24 h post-eclosion were injected with RNP complexes of BtKV-Cas9 and sgRNA without or with very low concentrations of saponin as endosomal agent, as application of higher concentrations inhibited transformation. *B. tabaci* females are capable of controlling offspring sex ratios and all survivors that made it to adulthood were male, which are haploid, so mutations are more likely to be observed in them. Despite that and after accounting for sex ratio bias, editing efficiency in whitefly females was approximately twofold higher than that of mosquitoes (Chaverra-Rodriguez *et al.*, 2018; Macias *et al.*, 2020). Germline editing was also confirmed by sequencing and genetic crosses, even though those did not follow classic Mendelian inheritance, with the trait being inherited at a rate less than the expected 50%. Below-Mendelian inheritance ratios are the result of fitness costs associated with carrying a *w* phenotype, which highlights the importance of ongoing research on more genes that display visual phenotypes upon knockdown and do not present such detrimental effects to the survival of the carrier. The successful application of ReMOT Control in *B. tabaci* opens the door for future genetic studies in such species that may shed light on candidates for potential development of species-specific biocontrol measures.

Nasonia vitripennis – parasitoid wasp

Contrary to *B. tabaci*, *Nasonia vitripennis*, a parasitic wasp of blowflies, is one of the most studied hymenopteran species and serves as a model insect in speciation (Breeuwer and Werren, 1995; Ellison *et al.*, 2008), sex ratio control (Werren, 1983; Parker and Orzack, 1985) and determination (Beukeboom and Kamping, 2006; Beukeboom *et al.*, 2007) and host–parasite evolution (Breeuwer and Werren, 1990; Bordenstein *et al.*, 2001). However, parasitic wasps and whiteflies share a haplodiploid sex-determination system where males develop from unfertilized eggs and are haploid (Werren and Loehlin, 2009), which is very advantageous to the screening of recessive mutations in genetic studies. CRISPR/Cas9 gene editing has been successfully used to mutate several genes that produce visible phenotypes in *Nasonia*, but its transformation is complex. *Nasonia* eggs must be dissected from host pupae prior to injection. The eggs are very small (0.08–1.16 mm) (Lalonde, 2005), their cytoplasm is extremely viscous, which frequently clogs the needle, and injected eggs require transplantation into a recipient blowfly pupa in order to develop properly (Li *et al.*, 2017). To determine whether ReMOT Control could be applied to the species, P2C-eGFP was injected into late-stage black pupae and efficient protein delivery into ovaries was seen at 1–3 days post injection (Chaverra-Rodriguez *et al.*, 2020). Toxicity of saponin was also tested, with no effects on survival of G_0 offspring at low concentrations. The approach to test gene editing through ReMOT was to deliver RNP complexes of P2C-Cas9 with an sgRNA targeting *Nasonia*'s *cinnabar* (*cin*) gene, which encodes kynerunine hydroxylase, an enzyme whose gene was previously targeted in mosquitoes (*kh*) and involved in the biosynthesis of the ommochromes required for dark eye pigmentation (Han *et al.*, 2003; Sethuraman and O'Brochta, 2005). Null mutations in *cin* (*cin⁻*) produce red-eye phenotypes (Li *et al.*, 2017) instead of the regular black (wild-type). The delivery of high concentrations of RNP complexes (about 3 μg/μl) resulted in 8.8% of the egg-laying females (4/45) producing independent *cin*-mutating events, where three G_0 individuals displayed a bright red-eye phenotype and one was mild red (Chaverra-Rodriguez *et al.*, 2020) (Fig. 6.2). To confirm editing of the genome, each of the G_0 *cin⁻* males was crossed to wild-type females and G_1 heterozygous females were allowed to lay haploid males from unfertilized eggs. G_2 male progeny presenting *cin⁻* phenotypes could only be generated if germline gene editing occurred in G_0, as was the case for the males with bright red eyes. No mild phenotypes were obtained in G_2, suggesting that the mild phenotype was due to somatic mosaicism (Chaverra-Rodriguez *et al.*, 2020).

Tetranychus urticae – spider mite

ReMOT Control has also been successful in chelicerates (Dermauw *et al.*, 2020). The two-spotted spider mite, *Tetranychus urticae*, is able to feed on 1100 different plant species, making it a very important herbivore pest worldwide that also developed resistance to more than 90 pesticides. Due to its extreme generalist diet, resistance to acaricides and having the smallest arthropod genome (90 Mb), it is regarded as a very good study model for adaptation (Grbić *et al.*, 2011; Wybouw *et al.*, 2015; Villarroel *et al.*, 2016; Bui *et al.*, 2018). Despite having its genome completely mapped, there are still challenges in developing reliable reverse genetics techniques to directly validate mutations in genes of interest. CRISPR/Cas9 approaches that rely on embryonic manipulation have not been successful in chelicerates, as embryos die upon microinjection (Garb *et al.*, 2018). ReMOT Control, however, relies on the injection of adults, a method that does not cause any associated extreme mortality in mites (about 75% survival). In this species, Cas9 was directly injected into adults without the use of a targeting ligand. *T. urticae* females were injected with RNP complexes that targeted the phytoene desaturase gene, which encodes an enzyme essential for red body pigmentation (Armstrong *et al.*, 1990). Albino males were recovered from the progeny after injection, albeit at very low numbers (0.4–0.6%) and some dying during development. One adult

male was recovered for each injection batch, isolated and crossed to obtain homozygous lines. All life stages for one of the lines (A) were completely albino (Fig. 6.2), whereas only immature stages (and adult legs) of the second line (B) displayed the phenotype (not shown). This difference is explained by the mutations generated in the germline of each of the lines. Failure to complement suggested that both lines harboured mutations in the targeted gene and sequencing confirmed it. Line A carried a 7 bp mutation, which caused a frameshift in the genomic locus and hence completely disrupted protein production. Line B, however, carried a 6 bp mutation, which deleted two amino acids but did not alter the protein frame, producing a hypomorphic mutation that only caused a partial loss of gene function and thus red could be partially seen in adult stages. Despite low transformation efficiencies, two independent events were induced in spider mites by RNP injections in adults, paving the way for future optimization of mutagenesis and transgenesis in the system. It is likely that the inclusion of an ovary-targeting ligand will improve the efficiency of spider mite genomic editing in the future.

Tribolium castaneum – red flour beetle

Tribolium castaneum, commonly known as the red flour beetle, is another species proven suitable for ReMOT Control. *T. castaneum* is a major pest of stored grain with a worldwide distribution that makes it incredibly economically relevant. Despite RNAi and transgenesis techniques being readily available for the species, there is a pressing need to expand the toolbox to less-specialized laboratories, as the organism has been used as an experimental model in genetics and developmental biology for decades. Recently, knockouts on *T. castaneum cardinal* (*cd*), an X-linked gene encoding for a haem peroxidase and involved in eye pigmentation, were achieved by injecting adult females using P2C-Cas9-sgRNA*cd* RNP complexes with two different sgRNA (Shirai and Daimon, 2020). The gene is evolutionarily conserved among insects and has been used as a marker for other novel gene-altering techniques such as population modification gene drives (Carballar-Lejarazú *et al.*, 2020). Upon injection of RNP complexes into 55 adult females, 34 of which survived, one *cd*- male was found in the progeny of those that laid (Fig. 6.2). This accounts for a mutagenesis efficiency of 3% (1/34), as one of the females presented a deletion, or of 0.2% in terms of hatched embryos (1/383) (Shirai and Daimon, 2020). Moreover, the *cd* knockout mutant presented a 4 bp deletion with undetectable wild-type sequence in its genome, suggesting that it did not present any mosaicism and, instead, the alteration of the gene occurred early in development during oogenesis (Shirai and Daimon, 2020). Both numbers and lack of mosaicism can be misleading, since they come from one particular experiment and minimal sample size, but ReMOT Control is a highly promising technique to be applied more and more regularly to the species.

Halyomorpha halys – brown marmorated stink bug case study

The power of ReMOT Control lies in its ease of use; any species where vitellogenic females can be injected can potentially be edited using the technique. Species do not have to be in culture in the laboratory; even material directly from the field can be edited. As a proof of principle, ReMOT Control was used to generate CRISPR-edited *Halyomorpha halys* (brown marmorated stink bug) directly from field-collected material. At the time, the stink bug genome had not yet been made available (Sparks *et al.*, 2020) and thus also proved that gRNAs designed from transcript data can be utilized for field-captured insects. *H. halys* is an Asian invasive species in the USA, Canada, Europe and South America (Leskey and Nielsen, 2018). The species causes severe crop loss to many specialty crops, including tree fruit, and its initial outbreak in the USA caused an economic loss of a calculated US$37 million in apples alone (Leskey *et al.*, 2012a). The species also has disrupted many integrated pest management programmes, leading to increased inputs of broad-spectrum insecticides such as pyrethroids and neonicotinoids (Leskey *et al.*, 2012b), which continue to be one of the only reliable control methods for the

species. *H. halys* is a member of the family Pentatomidae, which includes many problematic pest species worldwide and is thus a potentially beneficial group to consider for further genetic research. To investigate the capacity for CRISPR-mediated gene editing, the *H. halys kmo* orthologue was targeted using both embryo injections and ReMOT Control (Fig. 6.3). In embryo injections, which were performed using quartz needles injecting the Cas9 and sgRNA into the egg chorion, mutant phenotypes were found in 6/83 hatched nymphs. These were classified due to the apparent lack of total sclerotization across the whole body (*kmo⁻*) or only in one section of the pronotum (*kmo*^mos). Embryo injections, however, were time consuming and at times impractical due to several factors, including stink bug eggs being laid in clusters (which had to be carefully pulled apart for injections), difficult rearing, inconsistent egg laying, the use of an expensive microinjector, and egg chorion thickness causing continuous breakage of the needle. In parallel, the viability of ReMOT Control for *H. halys* was investigated. *H. halys* females were injected with a solution containing RNP complexes formed by sgRNAkmo and P2C-Cas9. Females were immobilized by gently placing a metal staple above the adult into a rubber block and pulling one wing to expose the thorax to allow for intrathoracic injection without harming the cuticle of the adult (Fig. 6.3). After injection, 11 independent egg masses were laid by the injected females and, out of those, 4/194 hatched nymphs displayed *kmo*^mos phenotypes (Fig. 6.2). This highlights the capacity of ReMOT Control to be applied for stink bug research with an efficiency similar to that observed with microembryonic injections and, importantly, empirically demonstrates that field material can be edited.

6.6   Challenges and Future Directions

Directed delivery of moieties to arthropod ovaries has a great potential to influence basic research on reproductive biology by creation of mutant and transgenic lines (reverse genetics) that in turn can be aimed to combat vector-borne disease, agricultural pests and insect conservation. A major step forward has been the development of a non-expensive method to deliver Cas9 RNPs to the ovaries that is not only widely usable in mosquito species, but is also being optimized for non-dipterans and species beyond insects. Despite that, some hurdles need to be overcome for the technique to be used to its full potential. Some of these hurdles exist for any kind of genetic manipulation across species, such as: (i) optimization of rearing; (ii) problems identifying mutants without visible phenotypes where high-throughput molecular identification (PCR) has to be performed; (iii) not all regions of the genome being equally accessible, especially for Cas9 cleavage (Jensen *et al*., 2017); and (iv) inefficiencies in HDR compared with other repair mechanisms that only incur random mutation of the targeted sequence (NHEJ, or microhomology-mediated end joining (MMEJ)) without insertion of a cargo. Others are challenges highly specific to each genus and sometimes even species. Among those, the most important features to account for are: (i) optimal timing of injection (especially for genera with long or uncharacterized oogenesis), both for survival of injected adults as well as successful DNA cleavage and repair upon translocation of the P2C-Cas9 RNPs; and (ii) capability of performing such injections without disrupting the laying capacity of females.

Despite ReMOT Control's high efficacy to translocate RNP complexes into the ovaries and to induce DNA cleavage, certain challenges are still present, the most pressing of which is the adaptation of the technique to generate targeted knock-ins. The biggest difficulty for this seems to be the delivery of template DNA, which would insert via HDR into the desired genomic cut site upon cleavage, to the ovaries. The template could theoretically be translocated in any DNA form (ssDNA or dsDNA, linearized or plasmid) or size (full or short-length homology arms in full plasmid, minicircle or short ssDNA), and certain mediators could be paired with the template to increase translocation and
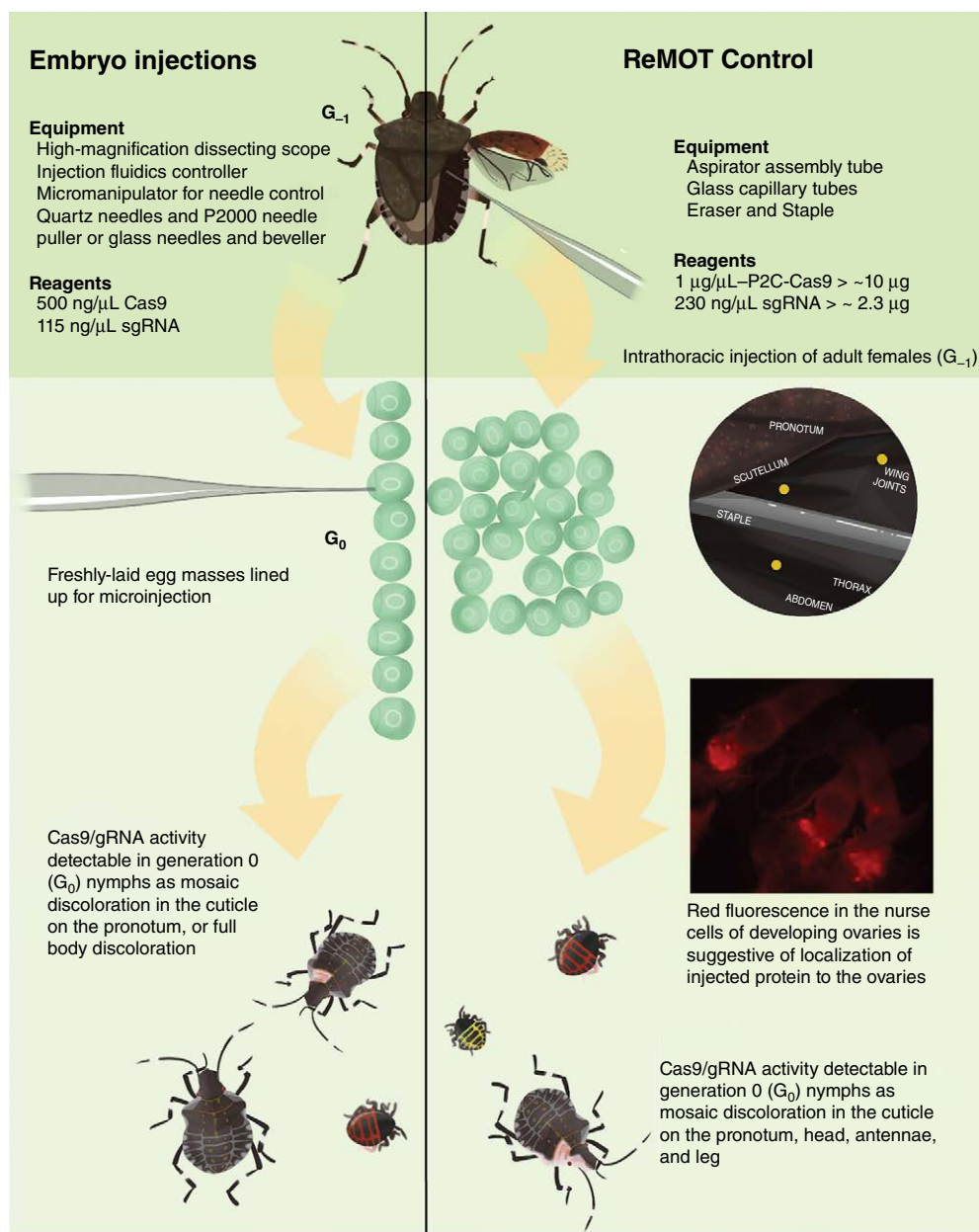
Fig. 6.3. Schematic model of ReMOT Control versus embryonic microinjection in the stink bug
H. halys. Both strategies are compared in terms of methodology (injection stage, required equipment and reagents). Generations (G_x) are indicated in each panel and separated using different shades of green.

proper delivery. This line of research is essential and ongoing and will expand, as the field would greatly benefit from a breakthrough. Another important aspect to bear in mind with ReMOT Control or other new genetic technologies is that many species lack transgenic or even genetic research because they have only become model species since the CRISPR revolution. This makes it harder to decide which genomic sequence to

target, because a marker is needed for a quick screening and visualization of knockout efficiency. As such, there is need to expand basic research on either phenotypes easy to visualize or in the generation of fluorescent transgenic lines.

6.7 Recommendations for Adaptation of ReMOT Control to New Species

One of the main advantages of using ReMOT Control over other techniques (i.e., embryonic microinjection) is that the transition to generating knockout lines for specific genes is less expensive, less specialized and appreciatively seamless. There are certain checkpoints that any new laboratory must examine to successfully create modified lines via ReMOT Control. The first is to test the ovarian uptake of a fluorescent fusion protein (e.g. P2C-GFP) to ensure that P2C or other targeting ligand of choice is able to mediate delivery to the oocytes. Researchers must be aware that some species display high levels of autofluorescence, so the appropriate controls must be used. If P2C does not work in a species of interest, a species-specific targeting ligand must be identified and validated (for example, BtKV for *B. tabaci*). Testing for protein uptake also allows for parallel assessment of survival of injected adults, as protocols may not have been developed for new models and some optimization may be required to deliver the injection mix into the haemolymph without harming the individual. Another component that can vary greatly between target species is the effectiveness of the different endosome escape reagents. Most species tolerate treatments containing saponin well (Chaverra-Rodriguez *et al.*, 2018) but if injected adults do not survive, an alternative chloroquine-based treatment (Li *et al.*, 2021), reduction to extremely low concentrations of saponin or even complete avoidance of escape agents can also be used, particularly as some of these reagents may be inhibitory in some species, for example saponin in *B. tabaci* (Heu *et al.*, 2020). Once a suitable injection protocol is developed, sgRNA(s) targeting the

gene of interest can be included in the mix. An important step that can be done beforehand is the identification of previously validated markers in the literature. Such markers are often associated with visual phenotypes involving pigmentation (see Fig. 6.2) of the eyes (e.g. *white* (Chaverra-Rodriguez *et al.*, 2018; Feng *et al.*, 2021); *cardinal* (Carballar-Lejarazú *et al.*, 2020; Feng *et al.*, 2021); *cinnabar* (Sethuraman and O'Brochta, 2005; Chaverra-Rodriguez *et al.*, 2020); *kmo* (Han *et al.*, 2003; Gantz *et al.*, 2015; Feng *et al.*, 2021)) or body (e.g. *yellow* (Feng *et al.*, 2021); *ebony* (Feng *et al.*, 2021)), most of them conserved throughout a range of species and genera. If none are available, a dominant non-lethal mutation presenting a visual phenotype is suitable for G_0 screening, as well as recessive genes in cases where there is a heterozygous mutant line. Dominant non-lethals are difficult to find, as most of the mutations are detrimental to fitness. If none of those described above is available, PCR screening at G_0 is required, which can be performed by amplification of the desired deletion followed by Sanger sequencing (for small deletions) or electrophoresis detection (if deletions are able to be visualized in a gel). A good trick for optimization for species that have such resources is to use (or make via HDR and embryonic microinjection) an established transgenic line containing a dominant fluorescent marker, as successful injection and mutagenesis of homozygous females (mated with non-fluorescent males) will produce non-fluorescent progeny.

6.8 Generalized ReMOT Control Protocol

This general protocol is designed around mosquitoes, but it can be adapted for any species of interest

6.8.1 Prior to ReMOT Control

Receptor/ligand

Identify the suitable receptor/ligand pair that allows for efficient Cas9 translocation

into the ovaries of the species of interest. While P2C has been shown to work in a broad range of distantly related species, it may not be the best candidate for certain organisms. Alternative candidates can be obtained from orthologous DmYP1/P2C sequences or other ligands derived from endogenous yolk protein precursors that can act as ovary transducers (see *B. tabaci* in section 6.5.2).

sgRNA

Generate sgRNAs from PCR template by following *in vitro* transcription protocols (Kistler *et al.*, 2015) (MegaScript T7 or RNAi kits). Two to four volumes of the standard *in vitro* transcription reactions using about 1 μg of PCR template were generally required to obtain enough sgRNA to perform adult injections. sgRNAs can also be purchased from vendors such as IDT, GenScript or Synthego, but this can be expensive. Store at –20°C or –80°C.

P2C-Cas9

Express and purify P2C-Cas9 (or other species-specific targeting ligand–Cas9 fusion) protein from plasmids transformed into recombinant BL21 *E. coli* (Chaverra-Rodriguez *et al.*, 2018). Dialyse the protein in a buffer consisting of 50 mM Tris-HCl pH 8.0, 200 mM KCl, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). Aliquot to avoid freeze–thaw degradation of protein. Store at –80°C. Depending on species, the protein may need to be buffer exchanged to low-salt, neutral pH buffer (see Macias *et al.*, 2020).

Adult insects

Rear G_1 insects to adulthood or collect and rear field-collected insects following standard laboratory procedures.

6.8.2   One day before injections

For anautogenous insects, blood-feed with a glass water-jacketed membrane feeder to induce vitellogenesis as, until that point,

oocyte development is in the previtellogenic arrest phase. For mosquitoes, ReMOT Control injections should be performed following a bloodmeal, but prior to completion of vitellogenesis (Chaverra-Rodriguez *et al.*, 2018; Macias *et al.*, 2020; Li *et al.*, 2021). Timing optimization will be needed for non-bloodsucking insects (use a targeting ligand–fluorescent protein fusion for this purpose), but injection has been shown to be efficient from late pupae to early adulthood (Chaverra-Rodriguez *et al.*, 2020; Heu *et al.*, 2020).

6.8.3   On injection day

For species in which dialysis buffer is intolerable for injection, buffer exchange should be performed prior to injection. P2C-Cas9 and similar fusion proteins were observed to precipitate when exchanged to low-salt, neutral pH buffers (Macias *et al.*, 2020; Li *et al.*, 2021); this does not seem to occur in the presence of nucleic acids, such as *in vitro* transcribed sgRNA or plasmid. On injection day, thaw a fresh aliquot of the P2C-Cas9 on ice and perform the following buffer exchange using the nucleic acid to be used during injection.

Prepare injection solutions

1. Dilute the total amount of P2C-Cas9 protein needed for the final injection mix in a large volume (300–500 μl) of 50 mM Tris-HCl pH 8.0 and 200 mM KCl.
2. Add the total amount of nucleic acids to be used in the injection at a smaller volume (10–20 μl) of highly concentrated nucleic acids; we generally use sgRNA (3–10 μg/μl). Incubate at room temperature for about 20 min to allow the RNP complex to form.
3. Buffer-exchange the solution with water (or desired neutral injection buffer) using an Amicon 10K filter column. The column is able to retain both the RNP complexes (sgRNA-bound P2C-Cas9) and unbound sgRNA molecules. By keeping track of the volume following each centrifugation and the volume of new buffer added at each centrifugation, the final salt concentration can be estimated.

For example, 300 μl total initial volume centrifuged to 40 μl followed by 360 μl new buffer will result in a new approximate KCl concentration of 20 mM.

4. When the concentration of the KCl is less than 5 mM and the volume is sufficiently small that the concentration of protein is above 0.5–0.75 μg/μl, remove the buffer-exchanged solution from the column and add water to the solution to obtain 0.5–0.75 μg P2C-Cas9/μl and around 1 μg total sgRNA/μl. Extra unbound sgRNA can be added if needed. Saponin (Chaverra-Rodriguez *et al.*, 2018; Macias *et al.*, 2020) (50 mg/l) and other endosome escape reagent can be added at this final step, even though in some cases they can have a detrimental effect on survival (Heu *et al.*, 2020).

Prepare adults

1. Immobilize G_1 females at 4°C and separate those with visible bloodmeals on an ice-cold Petri dish. Keep the soon-to-be injected mosquitoes on ice and discard the rest.
2. Inject blood-fed G_1 females intrathoracically using a glass capillary needle mounted on a mouth aspirator assembly (A5177, Sigma). Inject the mix solution containing P2C-RNP complexes until distention of the abdomen, diuresis or liquid release from the injection site can be observed. Injection protocols for non-mosquito species may have to be empirically optimized.
3. Place all injected G_1 females in cups, denoting the treatment and number of females injected to account for survival percentage.
4. Collect G_0 eggs as usual following laboratory procedures. Count the survival of G_0 larvae and screen for somatic cutting or phenotypic mosaicism. Outcross G_0 adults to obtain G_1 larvae (may display the knockout phenotype).

6.8.4 Screening protocol

G_0 and G_1 mosquitoes are aquatic at the immature stages and can be easily immobilized at the larval and pupal stages for dissection under a wide-field dissecting scope with or without fluorescence, depending on the marker phenotype. Mosquitoes can be strained in a flat sieve, but the size difference between L1 and L4 larvae is substantial, so care should be taken not to accidentally discard L1 larvae.

Immobilization is preferable using a vacuum pump, a Büchner funnel and 85 mm Whatman filter paper circles (Grade 1, Cat# 1001-085), which will allow retention of even very small stages, and the larvae and pupae can be kept wet during the screening process. The amount of time that the larvae can be kept on the wet filter paper varies by species, but usually 20 min does not seem to affect survival. Screening protocols for non-mosquito species must be empirically optimized.

1. Draw parallel lines roughly 1 cm apart across the filter paper; this will allow the screener to keep track of larvae screened under magnification.
2. Assemble the vacuum flask set-up by placing a Büchner funnel on top of a filtering flask using a rubber stopper or another material that can seal (e.g. modelling clay) and attach a tube to both the sidearm of the filtering flask and the vacuum source (e.g. portable vacuum pump or benchtop connection).
3. Place the lined filter paper onto the Büchner funnel; it should be bigger than the porous plate. Wet the paper and press it down so that the paper covers all the holes in the porous plate and comes up the sides of the funnel on the entire edge.
4. Add larvae/pupae to the funnel, taking care not to fill the water above the edge of the filter paper, and turn on the vacuum source to pull the water through.
5. Continue to add larvae and pupae as the water goes through until the filter paper is full. Up to about 500 larvae can be screened at a time, depending on the developmental stage. Every individual should be visible without overlapping.
6. Remove the moist paper with the larvae to a Petri dish for screening.
7. Fluorescence phenotypes and phenotypes visible to the naked eye can be visualized with the immobilization set-up described above

under a wide-field microscope with a plane that allows manual manipulation of the larvae/pupae with a paintbrush.

8. Mosquitoes should be monitored constantly to prevent drying, but should not be overly wet, since the increase in water increases the mobility of the larvae and makes their proper screening challenging. A transfer pipette can be used to add small amounts of water periodically. In contrast, a dry Whatman paper can be used to absorb excess water from the bottom of the lined paper during screening. Note that aquatic stages are sensitive to manipulation damage, so increased care should be taken when dealing with them.

6.8.5   *In vitro* protein expression protocol

This procedure is presented diagrammatically in Fig. 6.4.

Equipment requirements

1. Shaker with adjustable temperature. A shaker that can be refrigerated is preferable for expression of protein at 18°C, but proteins can also be expressed at a shorter incubation time at room temperature.

2. Sonicator (although alternative protocols for bacterial cell lysis without sonication are available).

3. Centrifuge capable of accommodating large volumes at speeds of up to 20,000 ×*g*.

BUFFERS.
- Wash buffer (20 mM Imidazole, 300 mM NaCl, 20 mM Tris-HCl pH 8.0).
- Lysis buffer (300 mM NaCl, 20 mM Imidazole, 20 mM Tris-HCl pH 8.0, 1 mg lysozyme/ml). EDTA-free protease inhibitors to be added immediately before use.
- Elution buffer (200 mM Imidazole, 300 mM NaCl, 20 mM Tris-HCl pH 8.0).
- Dialysis buffer (50 mM Tris-HCl pH 8.0, 200 mM KCl, 0.1 mM EDTA). Just before dialysis, add PMSF to a final concentration of 0.5 mM. 1 mM DTT in this solution is optional.

OTHER REAGENTS.
- Luria Broth (LB) agar for transformation.
- Selective antibiotic (kan, amp).
- Liquid Terrific Broth (TB). LB can also be used but may yield less protein.
- Invitrogen Ni-NTA Agarose beads.
- Isopropyl ß-d-1-thiogalactopyranoside (IPTG).
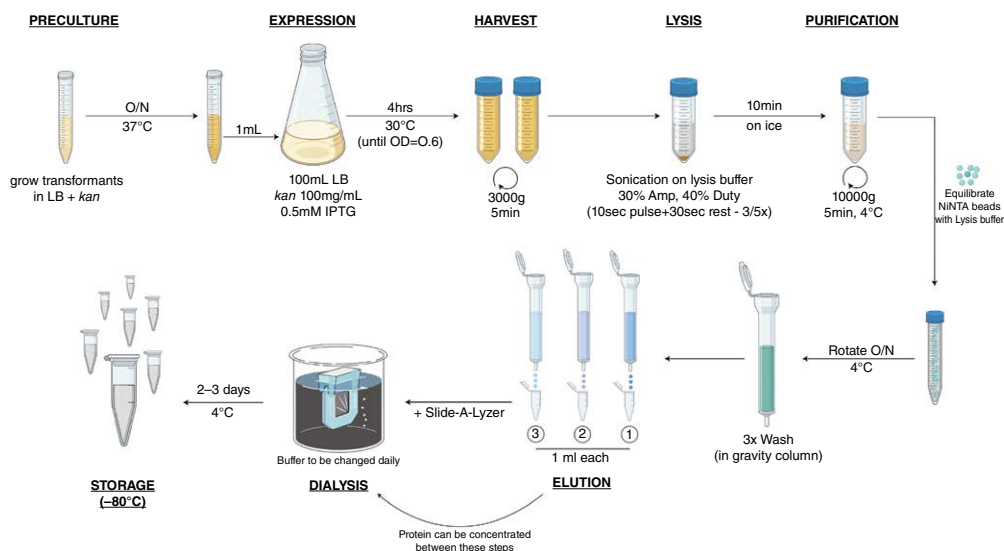
Fig. 6.4. Schematic representation of the protein generation protocol from a single plasmid-containing transformant colony to protein purification and storage.

Induction

1. Resuspend a single colony (from agar plate) or a stab (from glycerol) in 10–20 ml liquid culture (TB or LB with corresponding antibiotic).

2. Incubate at 37°C, shaking at 250–300 rpm overnight.

3. Inoculate 1 l of liquid medium with 10 ml of freshly grown culture. This can be scaled up and down proportionally (e.g. 3 ml in 300 ml of liquid medium). Incubate at 37°C with shaking at 250–300 rpm until OD600 reaches around 0.6 (0.4–0.8).

4. Induce protein expression for 4 h at 30°C by adding 4–40 μl of a 1 mM stock of IPTG for every 100 ml of culture (final concentration of 40–400 μM using optimal time/temperature determined in a small-scale trial). For the P2C-fusions proteins, use a final concentration of 400 μM.

5. Pellet cells at 6000 ×g for 15 minutes at 4°C, decant supernatant and either freeze the pellet at –80°C or proceed to lysis and purification.

Purification

1. Add 1 tablet of cOmplete™, EDTA-free protease inhibitor cocktail to 50 ml lysis buffer and shake until dissolved.

2. Resuspend pellet in about 40 ml cold lysis buffer. Keep on ice.

3. Sonicate (3–5x) using the following settings, if possible: 30% Amp, 40% Duty, 10s pulse, 30s rest.

4. Spin 30 min at 10,000 ×g at 4°C to collect the pellet. If the lysis was effective and the protein is soluble and not trapped in inclusion bodies, the protein should be in the lysate. In the meantime, equilibrate NiNTA agarose beads with lysis buffer. (These beads have nickel for binding of the protein his-tag. If your protein does not have a his-tag, use an alternative purification method.)

5. Add 1 ml (more or less) bead slurry from the bottle to a 15 ml conical tube.

6. Let beads settle at the bottom for about 5 min.

7. Remove the storage buffer using a pipette.

8. Add 1 ml lysis buffer and mix with beads.

9. Let beads settle again, and again remove the lysis buffer after they settle.

10. Remove supernatant from the lysed culture to 15 ml tubes with equilibrated NiNTA beads. Note: here we often take a small sample of the cell pellet to run on the final PAGE gel. This will allow you to see if any of the protein was in the pellet, which might indicate incomplete lysis or that the protein is trapped in inclusion bodies.

11. Bind protein and NiNTA beads by placing the tube containing the mix on the rotator at 4°C for at least 1 h, but an overnight incubation is typical.

12. At this step, there is often a substantial amount of cell debris that was not removed during the previous spin and the solution is often viscous. If this is the case, spin the mixture very briefly (seconds) at less than 1000 ×g, then remove the supernatant from the beads, add wash buffer to the beads and repeat. This will make the solutions move through the column more quickly.

13. Add beads to a column, one tube at a time until all the tubes for a given protein are loaded.

14. Wash with a column volume of cold wash buffer, either allowing the wash buffer to move through the column by gravity flow, or using a vacuum manifold. If a vacuum manifold is used, take care not to allow all of the liquid to pass through, which will cause the beads to dry.

15. Elute with successive 0.5 ml or 1 ml additions of cold elution buffer.

16. Run a few elutions, the first flowthrough, and the pellet sample on an SDS-PAGE gel. Coomassie stain and de-stain the gel to get an idea of what your purification and yield is.

Dialysis

1. Use Pall column 10 MWCO to concentrate pure protein to 3 ml or less.

2. Add the concentrated protein to a Slide-A-Lyzer™ 10,000 MWCO Cassette using a needle and syringe, taking special care not to touch the membrane either with your hands or with the needle.

3. Incubate at 4°C in 200-fold the amount of dialysis buffer with a spinner on the lowest spin speed, replacing the buffer each hour for 2 h (3 ml of concentrate should be incubated in 600 ml of dialysis buffer).

4. A final buffer replacement should be incubated at 4°C for incubation overnight.

5. Remove protein, aliquot, measure the concentration using the nanodrop or a Bradford assay and store at –80°C.

References

Adolfi, A., Gantz, V.M., Jasinskiene, N., Lee, H.-F., Hwang, K. *et al.* (2020) Efficient population modification gene-drive rescue system in the malaria mosquito *Anopheles stephensi*. *Nature Communications* 11, 5553.

Armstrong, G.A., Schmidt, A., Sandmann, G. and Hearst, J.E. (1990) Genetic and biochemical characterization of carotenoid biosynthesis mutants of *Rhodobacter capsulatus*. *Journal of Biological Chemistry* 265, 8329–8338.

Attardo, G.M., Hansen, I.A. and Raikhel, A.S. (2005) Nutritional regulation of vitellogenesis in mosquitoes: implications for anautogeny. *Insect Biochemistry and Molecular Biology* 35, 661–675.

Benoit, J.B., Attardo, G.M., Baumann, A.A., Michalkova, V. and Aksoy, S. (2015) Adenotrophic viviparity in tsetse flies: potential for population control and as an insect model for lactation. *Annual Review of Entomology* 60, 351–371.

Beukeboom, L.W. and Kamping, A. (2006) No patrigenes required for femaleness in the haplodiploid wasp *Nasonia vitripennis*. *Genetics* 172, 981–989.

Beukeboom, L.W., Kamping, A. and Van De Zande, L. (2007) Sex determination in the haplodiploid wasp *Nasonia vitripennis*: a critical consideration of models and evidence. *Seminars in Cell and Developmental Biology* 18, 371–378.

Bikard, D., Jiang, W., Samai, P., Hochschild, A., Zhang, F. *et al.* (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Research* 41, 7429–7437.

Bordenstein, S.R., O'Hara, F.P. and Werren, J.H. (2001) *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature* 409, 707–710.

Bownes, M., Hurd, H., Büsgen, T., Servay, D., Alvis, S. *et al.* (2002) *Drosophila* yolk protein produced in *E. coli* is accumulated by mosquito ovaries. *Insect Molecular Biology* 11, 487–496.

Breeuwer, J.A.J. and Werren, J.H. (1990) Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* 346, 558–560.

Breeuwer, J.A.J. and Werren, J.H. (1995) Hybrid breakdown between two haplodiploid species: the role of nuclear and cytoplasmic genes. *Evolution* 49, 705–717.

Bui, H., Greenhalgh, R., Ruckert, A., Gill, G.S., Lee, S. *et al.* (2018) Generalist and specialist mite herbivores induce similar defense responses in maize and barley but differ in susceptibility to benzoxazinoids. *Frontiers in Plant Science* 9.

Burt, A. (2003) Site-specific selfish genes as tools for the control and genetic engineering of natural populations. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 270, 921–928.

Carballar-Lejarazú, R., Ogaugwu, C., Tushar, T., Kelsey, A., Pham, T.B. *et al.* (2020) Next-generation gene drive for population modification of the malaria vector mosquito, *Anopheles gambiae*. *Proceedings of the National Academy of Sciences USA* 117(37), 22805–22814.

Catteruccia, F., Nolan, T., Blass, C., Müller, H.-M., Crisanti, A. *et al.* (2000) Toward *Anopheles* transformation: *Minos* element activity in anopheline cells and embryos. *Proceedings of the National Academy of Sciences USA* 97, 2157.

Chaverra-Rodriguez, D., Macias, V.M., Hughes, G.L., Pujhari, S., Suzuki, Y. *et al.* (2018) Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing. *Nature Communications* 9, 3008.

Chaverra-Rodriguez, D., Dalla Benetta, E., Heu, C.C., Rasgon, J.L., Ferree, P.M. *et al.* (2020) Germline mutagenesis of *Nasonia vitripennis* through ovarian delivery of CRISPR-Cas9 ribonucleoprotein. *Insect Molecular Biology* 29, 569–577.

Chen, Z., Jaafar, L., Agyekum, D.G., Xiao, H., Wade, M.F. *et al.* (2013) Receptor-mediated delivery of engineered nucleases for genome modification. *Nucleic Acids Research* 41, e182.

Cheng, A.W., Wang, H., Yang, H., Shi, L., Katz, Y. *et al.* (2013) Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Research* 23, 1163–1171.

Chouvenc, T. and Su, N.-Y. (2015) How do entomologists consume and produce their science? *American Entomologist* 61, 252–257.

Colosimo, A., Goncz, K.K., Holmes, A.R., Kunzelmann, K., Novelli, G. *et al.* (2000) Transfer and expression of foreign genes in mammalian cells. *Biotechniques* 29, 314–331.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R. *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819.

Cornel, A.J., Benedict, M.Q., Salazar Rafferty, C., Howells, A.J. and Collins, F.H. (1997) Transient expression of the *Drosophila melanogaster cinnabar* gene rescues eye color in the white eye (WE) strain of *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* 27**,** 993–997.

Czosnek, H., Hariton-Shalev, A., Sobol, I., Gorovits, R. and Ghanim, M. (2017) The incredible journey of begomoviruses in their whitefly vector. *Viruses* 9, 273.

Dermauw, W., Jonckheere, W., Riga, M., Livadaras, I., Vontas, J. *et al.* (2020) Targeted mutagenesis using CRISPR-Cas9 in the chelicerate herbivore *Tetranychus urticae*. *Insect Biochemistry and Molecular Biology* 120, 103347.

Dong, S., Lin, J., Held, N.L., Clem, R.J., Passarelli, A.L. *et al.* (2015) Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, *Aedes aegypti. PLoS ONE* 10, e0122353.

Dong, Y., Simões, M.L. and Dimopoulos, G. (2020) Versatile transgenic multistage effector-gene combinations for *Plasmodium falciparum* suppression in *Anopheles*. *Science Advances* 6, eaay5898.

Ellison, C.K., Niehuis, O. and Gadau, J. (2008) Hybrid breakdown and mitochondrial dysfunction in hybrids of *Nasonia* parasitoid wasps. *Journal of Evolutionary Biology* 21, 1844–1851.

Faria, M. and Wraight, S.P. (2001) Biological control of *Bemisia tabaci* with fungi. *Journal of Crop Protection* 20, 767–778.

Feng, X., Kambic, L., Nishimoto, J.H.K., Reed, F.A., Denton, J.A. *et al.* (2021) Evaluation of gene knockouts by CRISPR as potential targets for the genetic engineering of the mosquito *Culex quinquefasciatus*. *The CRISPR Journal* 4, 595–608.

FitzGerald, D.J., Trowbridge, I.S., Pastan, I. and Willingham, M.C. (1983) Enhancement of toxicity of anti-transferrin receptor antibody–*Pseudomonas* exotoxin conjugates by adenovirus. *Proceedings of the National Academy of Sciences USA* 80, 4134.

Fuchs, H., Bachran, D., Panjideh, H., Schellmann, N., Weng, A. *et al.* (2009) Saponins as tool for improved targeted tumor therapies. *Current Drug Targets* 10, 140–151.

Fuchs, H., Bachran, C. and Flavell, D.J. (2013) Diving through membranes: molecular cunning to enforce the endosomal escape of antibody-targeted anti-tumor toxins. *Antibodies* 2(2), 209–235.

Galizi, R., Doyle, L.A., Menichelli, M., Bernardini, F., Deredec, A. *et al.* (2014) A synthetic sex ratio distortion system for the control of the human malaria mosquito. *Nature Communications* 5, 3977.

Gantz, V.M. and Bier, E. (2015) The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. *Science* 348, 442–444.

Gantz, V.M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V.M. *et al.* (2015) Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. *Proceedings of the National Academy of Sciences USA* 112, E6736–E6743.

Garb, J.E., Sharma, P.P. and Ayoub, N.A. (2018) Recent progress and prospects for advancing arachnid genomics. *Current Opinion in Insect Science* 25, 51–57.

Gerling, D., Alomar, Ò. and Arnò, J. (2001) Biological control of *Bemisia tabaci* using predators and parasitoids. *Journal of Crop Protection* 20, 779–799.

Gilbert, L.A., Larson, M.H., Morsut, L., Liu, Z., Brar, G.A. *et al.* (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 154**,** 442–451.

Gilles, A.F., Schinko, J.B. and Averof, M. (2015) Efficient CRISPR-mediated gene targeting and transgene replacement in the beetle *Tribolium castaneum*. *Development* 142, 2832–2839.

Gratz, S.J., Cummings, A.M., Nguyen, J.N., Hamm, D.C., Donohue, L.K. *et al.* (2013) Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. *Genetics* 194, 1029.

Grbić, M., Van Leeuwen, T., Clark, R.M., Rombauts, S., Rouzé, P. *et al.* (2011) The genome of *Tetranychus urticae* reveals herbivorous pest adaptations. *Nature* 479, 487–492.

Grossman, G.L., Rafferty, C.S., Clayton, J.R., Stevens, T.K., Mukabayire, O. *et al.* (2001) Germline transformation of the malaria vector, *Anopheles gambiae*, with the piggyBac transposable element. *Insect Molecular Biology* 10, 597–604.

Guo, J.-Y., Wan, F.-H. and Ye, G.-Y. (2016) Oogenesis in the *Bemisia tabaci* MEAM1 species complex. *Micron* 83, 1–10.

Hall, D.G., Richardson, M.L., Ammar, E.-D. and Halbert, S.E. (2013) Asian citrus psyllid, *Diaphorina citri*, vector of citrus huanglongbing disease. *Entomologia Experimentalis et Applicata* 146, 207–223.

Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C. *et al.* (2016) A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nature Biotechnology* 34, 78–83.

Hammond, A., Pollegioni, P., Persampieri, T., North, A., Minuz, R. *et al.* (2021) Gene-drive suppression of mosquito populations in large cages as a bridge between lab and field. *Nature Communications* 12, 4589.

Han, Q., Calvo, E., Marinotti, O., Fang, J., Rizzi, M. *et al.* (2003) Analysis of the wild-type and mutant genes encoding the enzyme kynurenine monooxygenase of the yellow fever mosquito, *Aedes aegypti*. *Insect Molecular Biology* 12, 483–490.

Handler, A.M. and Harrell Ii, R.A. (1999) Germline transformation of *Drosophila melanogaster* with the piggyBac transposon vector. *Insect Molecular Biology* 8, 449–457.

Hege, K.M., Daleke, D.L., Waldmann, T.A. and Matthay, K.K. (1989) Comparison of anti-tac and anti-transferrin receptor-conjugated liposomes for specific drug delivery to adult T-cell leukemia. *Blood* 74, 2043–2052.

Heu, C.C., Mccullough, F.M., Luan, J. and Rasgon, J.L. (2020) CRISPR-Cas9-based genome editing in the silverleaf whitefly (*Bemisia tabaci*). *The CRISPR Journal* 3, 89–96.

Higgs, S., Olson, K.E., Klimowski, L., Powers, A.M., Carlson, J.O. *et al.* (1995) Mosquito sensitivity to a scorpion neurotoxin expressed using an infectious Sindbis virus vector. *Insect Molecular Biology* 4, 97–103.

Hilton, I.B., D'Ippolito, A.M., Vockley, C.M., Thakore, P.I., Crawford, G.E. *et al.* (2015) Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nature Biotechnology* 33, 510–517.

Huang, H.W., Chen, F.-Y. and Lee, M.-T. (2004) Molecular mechanism of peptide-induced pores in membranes. *Physical Review Letters* 92, 198304.

Isaacs, A.T., Li, F., Jasinskiene, N., Chen, X., Nirmala, X. *et al.* (2011) Engineered resistance to *Plasmodium falciparum* development in transgenic *Anopheles stephensi*. *PLoS Pathogens* 7, e1002017.

Jamison, B.V., Thairu, M.W. and Hansen, A.K. (2018) Efficacy of in vivo electroporation on the delivery of molecular agents into aphid ovarioles. *Journal of Insect Science* 18(2), 49.

Jasinskiene, N., Coates, C.J., Benedict, M.Q., Cornel, A.J., Rafferty, C.S. *et al.* (1998) Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the *Hermes* element from the housefly. *Proceedings of the National Academy of Sciences USA* 95, 3743.

Jasinskiene, N., Juhn, J. and James, A.A. (2007) Microinjection of *A. aegypti* embryos to obtain transgenic mosquitoes. *JoVE (Journal of Visualized Experiments)* 5, e219.

Jensen, K.T., Fløe, L., Petersen, T.S., Huang, J., Xu, F. *et al.* (2017) Chromatin accessibility and guide sequence secondary structure affect CRISPR-Cas9 gene editing efficiency. *FEBS Letters* 591, 1892–1901.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. *et al.* (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821.

Jinek, M., East, A., Cheng, A., Lin, S., Ma, E. *et al.* (2013) RNA-programmed genome editing in human cells. *eLife* 2, e00471.

Kistler, K.E., Vosshall, L.B. and Matthews, B.J. (2015) Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*. *Cell Reports* 11, 51–60.

Kokoza, V., Ahmed, A., Wimmer, E.A. and Raikhel, A.S. (2001) Efficient transformation of the yellow fever mosquito *Aedes aegypti* using the piggyBac transposable element vector pBac [3xP3-EGFP afm]. *Insect Biochemistry and Molecular Biology* 31, 1137–1143.

Kravariti, L., Thomas, J.L., Sourmeli, S., Rodakis, G.C., Mauchamp, B. *et al.* (2001) The biolistic method as a tool for testing the differential activity of putative silkmoth chorion gene promoters. *Insect Biochemistry and Molecular Biology* 31, 473–479.

Kyrou, K., Hammond, A.M., Galizi, R., Kranjc, N., Burt, A. *et al.* (2018) A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged *Anopheles gambiae* mosquitoes. *Nature Biotechnology* 36, 1062–1066.

Labbé, G.M.C., Nimmo, D.D. and Alphey, L. (2010) piggybac- and phiC31-mediated genetic transformation of the Asian tiger mosquito, *Aedes albopictus. PLoS Neglected Tropical Diseases* 4, e788.

Lalonde, R.G. (2005) Egg size variation does not affect offspring performance under intraspecific competition in *Nasonia vitripennis*, a gregarious parasitoid. *Journal of Animal Ecology* 74, 630–635.

Leskey, T.C. and Nielsen, A.L. (2018) Impact of the invasive brown marmorated stink bug in North America and Europe: history, biology, ecology, and management. *Annual Review of Entomology* 63, 599–618.

Leskey, T.C., Hamilton, G.C., Nielsen, A.L., Polk, D.F., Rodriguez-Saona, C. *et al.* (2012a) Pest status of the brown marmorated stink bug, *Halyomorpha halys* in the USA. *Outlooks on Pest Management* 23, 218–226.

Leskey, T.C., Lee, D.-H., Short, B.D. and Wright, S.E. (2012b) Impact of insecticides on the invasive *Halyomorpha halys*: analysis of insecticide lethality. *Journal of Economic Entomology* 105, 1726–1735.

Leta, S., Beyene, T.J., De Clercq, E.M., Amenu, K., Kraemer, M.U.G. *et al.* (2018) Global risk mapping for major diseases transmitted by *Aedes aegypti* and *Aedes albopictus*. *International Journal of Infectious Diseases* 67, 25–35.

Li, F., Zhao, X., Li, M., He, K., Huang, C. *et al.* (2019) Insect genomes: progress and challenges. *Insect Molecular Biology* 28, 739–758.

Li, M., Au, L.Y.C., Douglah, D., Chong, A., White, B.J. *et al.* (2017) Generation of heritable germline mutations in the jewel wasp *Nasonia vitripennis* using CRISPR/Cas9. *Science Reports* 7, 901.

Li, M., Yang, T., Kandul, N.P., Bui, M., Gamez, S. *et al.* (2020) Development of a confinable gene drive system in the human disease vector *Aedes aegypti*. *eLife* 9, e51701.

Li, X., Xu, Y., Zhang, H., Yin, H., Zhou, D. *et al.* (2021) ReMOT Control delivery of CRISPR-Cas9 ribonucleoprotein complex to induce germline mutagenesis in the disease vector mosquitoes *Culex pipiens pallens*. *Journal of Medical Entomology* 58, 1202–1209.

Liang, W. and Lam, J.K.W. (2012) Endosomal escape pathways for non-viral nucleic acid delivery systems. Chapter 17 in: Ceresa, B. (ed.) *Molecular Regulation of Endocytosis.* IntechOpen, London.

López Del Amo, V., Bishop, A.L., Sánchez C.H.M., Bennett, J.B., Feng, X. *et al.* (2020) A transcomplementing gene drive provides a flexible platform for laboratory investigation and potential field deployment. *Nature Communications* 11, 352.

Lule-Chávez, A.N., Carballar-Lejarazú, R., Cabrera-Ponce, J.L., Lanz-Mendoza, H. and Ibarra, J.E. (2021) Genetic transformation of mosquitoes by microparticle bombardment. *Insect Molecular Biology* 30, 30–41.

Macias, V.M., Mckeand, S., Chaverra-Rodriguez, D., Hughes, G.L., Fazekas, A. *et al.* (2020) Cas9-mediated gene-editing in the malaria mosquito *Anopheles stephensi* by ReMOT Control. *G3 (Bethesda)* 10, 1353–1360.

Maeda, S., Kawai, T., Obinata, M., Fujiwara, H., Horiuchi, T. *et al.* (1985) Production of human α-interferon in silkworm using a baculovirus vector. *Nature* 315, 592–594.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M. *et al.* (2013) RNA-guided human genome engineering via Cas9. *Science* 339, 823.

Matthews, B.J. and Vosshall, L.B. (2020) How to turn an organism into a model organism in 10 'easy' steps. *Journal of Experimental Biology* 223 (Pt Suppl. 1), jeb 218198.

Miles, A., Harding, N.J., Bottà, G., Clarkson, C.S., Antão, T. *et al.* (2017) Genetic diversity of the African malaria vector *Anopheles gambiae*. *Nature* 552, 96–100.

Miller, L.H., Sakai, R.K., Romans, P., Gwadz, R.W., Kantoff, P. *et al.* (1987) Stable integration and expression of a bacterial gene in the mosquito *Anopheles gambiae*. *Science* 237, 779.

Mojica, F.J.M. and Rodriguez-Valera, F. (2016) The discovery of CRISPR in archaea and bacteria. *FEBS Journal* 283, 3162–3169.

Morgan, T.H. (1910) Sex limited inheritance in *Drosophila*. *Science* 32, 120.

Nimmo, D.D., Alphey, L., Meredith, J.M. and Eggleston, P. (2006) High efficiency site-specific genetic engineering of the mosquito genome. *Insect Molecular Biology* 15, 129–136.

Noah Koller, C., Dhadialla, T.S. and Raikhel, A.S. (1989) Selective endocytosis of vitellogenin by oocytes of the mosquito, *Aedes aegypti*: an *in vitro* study. *Insect Biochemistry* 19, 693–702.

Parker, E.D. Jr and Orzack, S.H. (1985) Genetic variation for the sex ratio in *Nasonia vitripennis*. *Genetics* 110, 93–105.

Pham, T.B., Phong, C.H., Bennett, J.B., Hwang, K., Jasinskiene, N. *et al.* (2019) Experimental population modification of the malaria vector mosquito, *Anopheles stephensi. PLoS Genetics* 15, e1008440.

Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S. *et al.* (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152, 1173–1183.

Qian, Z.M., Li, H., Sun, H. and Ho, K. (2002) Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. *Pharmacological Review* 54, 561.

Raikhel, A.S. (1984) The accumulative pathway of vitellogenin in the mosquito oocyte: a high-resolution immuno- and cytochemical study. *Journal of Ultrastructure Research* 87, 285–302.

Sethuraman, N. and O'Brochta, D.A. (2005) The *Drosophila melanogaster* cinnabar gene is a cell autonomous genetic marker in *Aedes aegypti*. *Journal of Medical Entomology* 42, 716–718.

Shirai, Y. and Daimon, T. (2020) Mutations in cardinal are responsible for the red-1 and peach eye color mutants of the red flour beetle *Tribolium castaneum*. *Biochemical and Biophysical Research Communications* 529, 372–378.

Simoni, A., Hammond, A.M., Beaghton, A.K., Galizi, R., Taxiarchi, C. *et al.* (2020) A male-biased sex-distorter gene drive for the human malaria vector *Anopheles gambiae*. *Nature Biotechnology* 38, 1054–1060.

Smiseth, P.T., Ward, R.J.S. and Moore, A.J. (2006) Asynchronous hatching in *Nicrophorus vespilloides*, an insect in which parents provide food for their offspring. *Functional Ecology* 20, 151–156.

Souza-Neto, J.A., Powell, J.R. and Bonizzoni, M. (2019) *Aedes aegypti* vector competence studies: a review. *Infection, Genetics and Evolution* 67, 191–209.

Sparks, M.E., Bansal, R., Benoit, J.B., Blackburn, M.B., Chao, H. *et al.* (2020) Brown marmorated stink bug, *Halyomorpha halys*, genome: putative underpinnings of polyphagy, insecticide resistance potential and biology of a top worldwide pest. *BMC Genomics* 21, 227.

Stavridis, J.C. and Psallidopoulos, M. (1982) Use of transferrin as a gene-carrier to the erythroid cells of the marrow. *Cellular and Molecular Biology* 28, 15–18.

Terradas, G., Buchman, A.B., Bennett, J.B., Shriner, I., Marshall, J.M. *et al.* (2021) Inherently confinable split-drive systems in *Drosophila*. *Nature Communications* 12, 1480.

Thomas, J.-L. (2003) Electroporation, an alternative to biolistics for transfection of *Bombyx mori* embryos and larval tissues. *Journal of Insect Science* 3, 17.

Villarroel, C.A., Jonckheere, W., Alba, J.M., Glas, J.J., Dermauw, W. *et al.* (2016) Salivary proteins of spider mites suppress defenses in *Nicotiana benthamiana* and promote mite reproduction. *Plant Journal* 86, 119–131.

Wagner, E., Curiel, D. and Cotten, M. (1994) Delivery of drugs, proteins and genes into cells using transferrin as a ligand for receptor-mediated endocytosis. *Advanced Drug Delivery Reviews* 14, 113–135.

Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W. *et al.* (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153, 910–918.

Werren, J.H. (1983) Brood size and sex ratio regulation in the parasitic wasp *Nasonia vitripennis*. *Netherlands Journal of Zoology* 34, 123–143.

Werren, J.H. and Loehlin, D.W. (2009) The parasitoid wasp *Nasonia*: an emerging model system with haploid male genetics. *Cold Spring Harbor Protocols* 2009, pdb.emo134–pdb.emo134.

Widera, A., Norouziyan, F. and Shen, W.C. (2003) Mechanisms of TfR-mediated transcytosis and sorting in epithelial cells and applications toward drug delivery. *Advanced Drug Delivery Reviews* 55, 1439–1466.

Wu, M. (1997) Enhancement of immunotoxin activity using chemical and biological reagents. *British Journal of Cancer* 75, 1347–1355.

Wybouw, N., Zhurov, V., Martel, C., Bruinsma, K.A., Hendrickx, F. *et al.* (2015) Adaptation of a polyphagous herbivore to a novel host plant extensively shapes the transcriptome of herbivore and host. *Molecular Ecology* 24, 4647–4663.

7 Site-Directed DNA Sequence Modification Using CRISPR/Cas 9

Carolina Concha[1]* and Riccardo Papa[2]
[1]*Smithsonian Tropical Research Institute, Panama City, Panama;* [2]*Department of Biology, Universidad de Puerto Rico, San Juan, Puerto Rico*

7.1 The CRISPR/Cas9 Revolution

Ever since the discovery of the DNA double helix, researchers have contemplated the possibility of making site-specific changes to the genomes of cells and organisms. When cellular mechanisms of DNA repair and recombination were unravelled (Rudin *et al.*, 1989), scientists reasoned that genomic changes could be introduced by using sequence-specific nucleases to create targeted breaks in the genome and then exploiting the cells' natural repair mechanisms to restore the damage (Thomas *et al.*, 1986). Using this approach, gene editing started with the finding of homing endonucleases in the genomes of yeast and many microorganisms (Colleaux *et al.*, 1986; Dujon, 1989). These enzymes are often encoded within introns and their genes are embedded within the enzyme's own cleavage recognition sequence such that they cut genomic sequences lacking the intron, for example sister chromatids, stimulating the cellular recombination and repair processes to fix the break by simply copying the gene encoding them into the broken chromosome (Jacquier and Dujon, 1985). Researchers exploited this system by inserting desired sequences into the intron so that they would be integrated into the genome at sites recognized by the homing endonuclease (Chevalier *et al.*, 2002). Contemporaneously, the zinc-finger nucleases (ZFNs) were created (Kim *et al.*, 1996; Bibikova *et al.*, 2002). These enzymes consist of modular DNA recognition proteins called zinc fingers that are coupled to the cleavage domain of the *Fok*I nuclease and, as such, can function as site-specific nucleases. These nucleases can be engineered to recognize a specific DNA region of interest and were found to be very efficient at inducing genomic changes in *Drosophila* and mammalian cells (Bibikova *et al.*, 2003). More recently, another gene editing system used small proteins discovered in bacteria which bind single nucleotides, named transcription activator-like effectors (TALEs), coupled to the cleavage domain of *Fok*I, to create transcription activator-like effector nucleases (TALENs) (Bogdanove and Voytas, 2011). Similar to the ZFN, several TALEs are engineered together so that they bind to a desired target sequence. Although homing endonucleases, ZFNs and TALENS are effective genome editing tools, they were not widely adopted across the research community because of the difficulty in designing

---

*Corresponding author, email: carolacmcl@yahoo.com

and optimizing the systems for each specific genomic target, which made them labour intensive and costly.

In this chapter we provide a background on genome editing approaches in insects, including advantages and disadvantages of specific applications. By presenting clear evidence of the use of a variety of methodologies, we take the opportunity to highlight some of the critical aspects that ensure the success of such approaches. Finally, we contextualize these techniques within a research backdrop, displaying their use to answer scientific hypotheses.

7.1.1 CRISPR/Cas systems in bacterial immunity

In the late 1980s Japanese microbiologists observed a series of short direct repeats interspaced with short sequences in the genome of the bacterium *Escherichia coli*, which they named CRISPRs (clustered regular interspaced short palindromic repeats) (Ishino *et al*., 1987). The function of these sequences was not understood at the time and remained unknown until the mid-2000s, when further studies showed that these repeats were also found in other bacteria and archaea (Mojica *et al*., 2000). Indeed, many of the spacer sequences they contained were found to be derived from plasmids or viruses (Bolotin *et al*., 2005; Mojica *et al*., 2005). These observations, together with the findings that the CRISPR locus is transcribed and that it contains Cas (CRISPR-associated) genes, which encode proteins with potential nuclease and helicase activities, gave the first hints about what the function of these repeats was: an adaptive immune system that enables bacteria to defend themselves against infections (Barrangou *et al*., 2007; Garneau *et al*., 2010).

The bacterial CRISPR/Cas locus is composed of an operon of *cas* genes encoding the Cas proteins and an array of identical repeats alternated with short 'invader' DNA sequences, called spacers, which provide a chronological history of the viruses and plasmids that have invaded a given bacterial strain (Fig. 7.1A). The CRISPR immune defence occurs in three steps: adaptation, expression and interference (Barrangou and Marraffini, 2014). In the adaptation step, fragments of invading DNA are incorporated in the CRISPR array as spacers. During expression, transcription from the CRISPR locus generates a precursor CRISPR RNA (pre-crRNA) that is subsequently processed into individual mature crRNAs, composed of a repeat portion and an invader-targeting spacer portion (Fig. 7.1B). In the final interference step, invading DNA is cleaved by a crRNA-guided Cas enzyme at a site complementary to the spacer sequence (Fig. 7.1C).

The evolution of the invader–host relationship between bacteria and their infectious viruses has given rise to three CRISPR/Cas systems which differ in the molecular mechanisms of DNA recognition and cleavage (Makarova *et al*., 2011; van der Oost *et al*., 2014). In type I and type II systems, the specific recognition of foreign DNA from bacterial genomic sequences requires the presence of a protospacer adjacent motif (PAM) in the invader's genome, which is a short sequence adjacent to the target sequence to which the Cas enzyme binds (Shah *et al*., 2013). A special feature of type II systems is that they use two RNA molecules for recognition and cleavage of invader DNA. Indeed, this system requires a *trans*-activating crRNA (tracrRNA), a small RNA molecule that is *trans* encoded upstream of the type II CRISPR locus and that plays a role in maturation of the crRNA. The tracrRNA is partially complementary to and binds the pre-crRNA forming an RNA duplex, which is cleaved by RNAse III to form a mature duplex that acts as a guide for the Cas protein to cleave a specific sequence in the invading nucleic acid (Fig. 7.1B). The type I and type III CRISPR/Cas systems use a large complex of Cas proteins for recognition and cleavage of foreign DNA, whereas the type II system requires only a single Cas enzyme for specific genomic cleavage. Furthermore, the best-studied Cas effector protein is Cas9 from *Streptococcus pyogenes*, a large enzyme with two nuclease domains that introduces dsDNA breaks in invading DNA at a site within a specific 20-nucleotide (nt) sequence complementary to the crRNA and

(A)   Genomic CRISPR locus
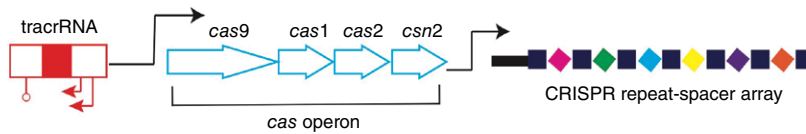
(B)   tracrRNA:crRNA co-maturation and Cas9 co-complex formation
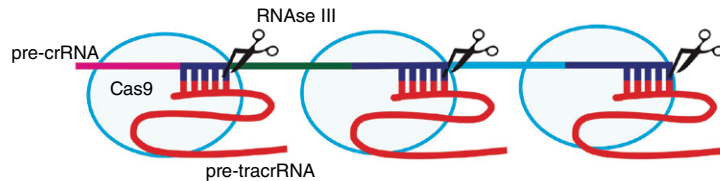
(C)   RNA-guided cleavage of target DNA
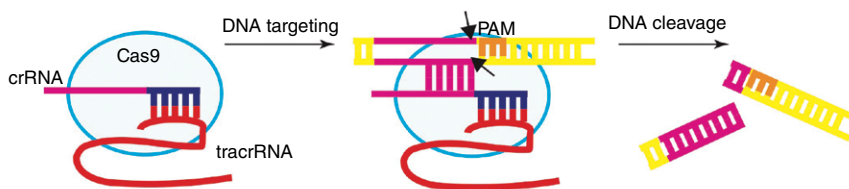
(D)   CRISPR-cas9 genome editing
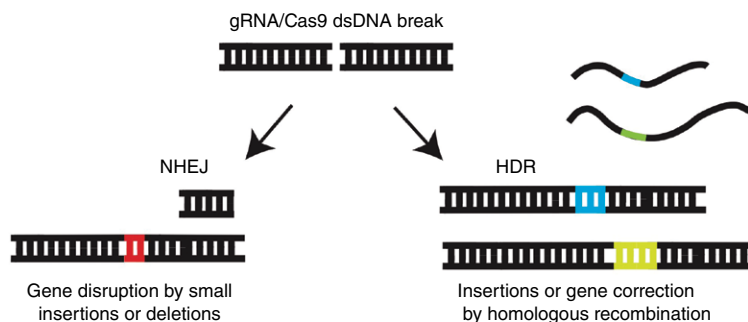
Fig. 7.1. CRISPR-Cas9 is a bacterial immune defence system and has been co-opted as a genome engineering tool. (A) Genomic CRISPR locus from *S. pyogenes* containing the operon of *cas* genes and the CRISPR array of identical sequence repeats (blue boxes) alternated with short invader DNA sequences (coloured diamonds). Upstream of the Cas operon, we find the tracrRNA locus. **(B)** The bacterial antiviral defence involves association of the Cas9 protein with precursor tracrRNA:crRNA duplexes followed by co-processing of the RNA by Ribonuclease III. **(C)** Mature Cas9-tracrRNA:crRNA complexes bind target viral DNA at a site complementary to the crRNA and adjacent to a PAM sequence, where Cas9 creates a dsDNA break. **(D)** Cas9/gRNA cleavage of genomic DNA is repaired by the cellular DNA repair mechanisms: either non-homologous end joining (NHEJ), an error-prone process that introduces mutations or deletions, or homology-directed repair (HDR), a process that uses a DNA molecule as template for repair. As a genome editing tool, we take advantage of these two DNA repair mechanisms to create mutations, deletions and insertions in a chosen cell or organism. Diagrams adapted from Doudna and Charpentier (2014).

adjacent to the PAM (Gasiunas *et al.*, 2012; Jinek *et al.*, 2012). These characteristics make bacterial CRISPR/Cas type II systems a very attractive tool to be adopted for use in genome engineering in the laboratory.

7.1.2 CRISPR/Cas9 as a genome editing tool

The CRISPR/Cas9 genome editing tool is a simple two component system that includes the Cas9 enzyme and a single-guide RNA molecule (sgRNA), which is an engineered shorter version of the mature crRNA/tracrRNA hybrid. The sgRNA contains a 5′ 20 nt sequence complementary to the target genomic DNA and a 3′ double-stranded RNA structure that facilitates binding to the Cas9 enzyme (Jinek *et al.*, 2012). The Cas9 enzyme and the guide RNA (gRNA) form a complex in which the gRNA directs the enzyme to the cleavage site in the target DNA sequence, where the Cas9 enzyme performs a dsDNA break at a location in the complementary sequence close to the PAM (Cong *et al.*, 2013). The great advantage of this system is that by simply changing the specific 20 nt sequence in the gRNA we can direct CRISPR/Cas9 to perform dsDNA cleavage of any target DNA sequence as long as it is adjacent to a PAM, making it a very versatile genome editing tool (Doudna and Charpentier, 2014).

The main practical uses of CRISPR/Cas9 are making mutations, deletions and insertions into the desired target genomes by relying on its ability to make site-directed dsDNA breaks and then allowing the cells to sense the break and respond via one of two possible DNA repair pathways: non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Wyman and Kanaar, 2006) (Fig. 7.1D). Indeed, when cells repair DNA damage via NHEJ they tend to introduce DNA changes in a few nucleotides near the cut site with high frequency, which can be exploited to generate site-directed mutations. Moreover, if two or more gRNAs are used with Cas9 simultaneously to target sites relatively close to each other, it is possible to use NHEJ to create desired site-specific deletions in the genome as a result of the removal of a DNA fragment in between the cut sites. These strategies can be used to generate open reading frame (ORF) mutations in genes to create knockout phenotypes, or deleting whole genes, gene exons or gene regulatory elements, all of which are useful for the study of gene function and regulation (Sander and Joung, 2014). Furthermore, it is possible to use CRISPR/Cas9 to generate insertions in the genome of a given organism by creating one or two dsDNA breaks and then taking advantage of either the NHEJ (Farnworth *et al.*, 2020) or the HDR pathway to repair the damage (Gratz *et al.*, 2014) (see Ahmed and Wimmer, Chapter 5, this volume). Simply by providing a single- or double-stranded DNA template with homology to the target DNA sequence along with the Cas9/gRNA complexes, we can leverage the HDR machinery for inserting a desired DNA fragment at the chosen cut site (Gratz *et al.*, 2013). This strategy can be successfully used to change small sequences in the genome for a desired sequence, for example to correct a mutation or to create a precise mutation of choice, or it can be used to insert longer DNA fragments or whole genes to create knock-in phenotypes (see Ahmed and Wimmer, Chapter 5, this volume).

Since the co-opting of the CRISPR/Cas9 system into a genetic engineering tool (Jinek *et al.*, 2012), it was gradually adopted for a wide array of applications, from genome editing of cultured mammalian cells and cell lines to germline modification of whole organisms, until it became a routine technique in the laboratory (Bassett and Liu, 2014a,b). The simplicity of its design and ease of use, together with its low cost and high efficiency, make it possible for the technology to be established in any molecular biology laboratory. The impact that this has had in the scientific research community in such a short time has earned Jennifer Doudna and Emmanuelle Charpentier, the scientists who led these developments, the Nobel Prize in Chemistry in 2020 (Ledford and Callaway, 2020). In the field of insect genetics and biotechnology, this system has been widely adopted for many different applications.

7.2   Site-Directed Genomic Modifications in Insects (Version 2.0)

The first attempts at introducing genomic mutations in insects came with chemical and radiation mutagenesis, where random DNA breaks would be created and then repaired by the cellular machinery (Sobels, 1985; Sekelsky, 2017). With this approach, insect mutants had to be identified after the experiment and characterized for genomic modifications. Later, transgenesis methods were developed using transposable elements to introduce large DNA fragments into the genome (Gloor *et al.*, 1991, reviewed in Handler and Atkinson, 2006) (see O'Brochta, Chapter 1, this volume). This strategy has been widely used to create transgenic insects and many insect transposable elements have been isolated and tested for this purpose (reviewed in Fraser, 2012). However, the disadvantage of this method is that insertions in the genome are created randomly, without any site-directed control, and they must be screened for their number and position in the genome. Furthermore, the expression of inserted genes or gene cassettes is often affected by the environment of their insertion site within the genome (Wallrath and Elgin, 1995) and the resulting germline mutants must also be tested for variation in the level of expression and activity of the inserted genes. Moreover, the presence of transposable element recognition sequences in the genome of the engineered insects has the potential to re-mobilize the whole insertion cassette to a different location of the genome if they encounter insect transposases, introducing a risk of instability of the transgenes (Hoy, 2000; Handler, 2004). These challenges encouraged the development of site-directed technologies of insect genome engineering. Indeed, endonuclease-based technologies like homing endonucleases, ZNFs and TALENs have been used in insects with good success (Bibikova *et al.*, 2002; Yu *et al.*, 2014), but due to the considerable effort and cost involved in engineering the endonucleases for each specific target, they have not been widely adopted.

With the recent development of CRISPR/Cas9 technology we have witnessed a surge in genome engineering of insects for many different applications. A detailed description of the steps of CRISPR/Cas9 experiments in insects has been explored in several reviews (Bassett and Liu, 2014a,b; Housden *et al.*, 2014; Gratz *et al.*, 2015; Bier *et al.*, 2018). Here, we present the basics of performing targeted mutations and deletions with CRISPR/Cas9 genome editing in insects, focusing only on the most relevant aspects of designing, performing and interpreting the result of an experiment.

7.2.1   Designing sgRNA

The design of a genome editing experiment is the first step, and an extremely important one, as it impacts the success or failure of the whole experiment. In order to design effective sgRNAs, a good-quality reference genome is needed. If the objective is to target a coding sequence, then having a reference genome should be paired with a transcriptome annotation of good coverage across genes. While coding sequences tend to be very conserved within individuals of the same species and closely related species, non-coding sequences can be highly variable even within the same population. Thus, if the objective is to target a non-coding sequence like an intron or a regulatory region, it would be recommended to have sequencing data for at least 10 or 20 individuals of the same species or population. This information can be used to make a multiple sequence alignment across the region of interest and select a conserved region for the design of reliable sgRNAs. Another important aspect that should be considered is the uniqueness of the genomic target sequence in order to avoid deleterious off-target effects. Since the Cas9 nuclease is known to tolerate some mismatches between sgRNA and the target genome, it is recommended to run a BLAST search of the designed sgRNA against the complete genome in order to avoid target sites occurring very frequently. Ideally, an sgRNA should be selected with

the closest off-target site differing by at least 4 nt (Bassett and Liu, 2014a,b). However, mutations located towards the 3′ end of the sgRNA, near the PAM, are not tolerated by Cas9 and may prevent cleavage.

Generally, for the design of sgRNAs the objective is to select a 20 nt sequence that precedes an NGG sequence, or PAM, in the genome (Jinek *et al*., 2012). This is the only strict sequence requirement for sgRNA design and both strands of genomic DNA can be targeted. There is a great level of variability in the efficiency of different sgRNAs, probably due to secondary structure formation in the RNA or variation in the accessibility of the DNA sequence within chromatin (Bassett and Liu, 2014a,b; Farboud and Meyer, 2015). For this reason, in order to increase the chances of good genome editing, it is useful to design between two and four sgRNA for each genomic target. Moreover, several studies in other organisms have suggested that sgRNAs that cleave most efficiently have low U content and a high GC content within the six nucleotides before the PAM (Farboud and Meyer, 2015; Malina *et al*., 2015; Moreno-Mateos *et al*., 2015; Labuhn *et al*., 2018). All these considerations should be integrated into the design of the sgRNAs that will be used. Several websites and web-based software are available to help design sgRNA that minimize the chances of having off-target effects (reviewed in Yennmalli *et al*., 2017). Additionally, although SpCas9 is the most widely used nuclease for genome editing, with a PAM recognition sequence 5′-NGG-3′, other CRISPR type II enzymes have been investigated for dsDNA cleavage which recognize different PAM sequences, thereby giving a broader range of options for the design of sgRNA against a target genomic DNA. For example, Cas12a from *Lachnospiraceae bacterium* (previously known as Cpf1) recognizes a 5′-TTTN-3′ sequence as PAM and has been tested in *Drosophila* with some success, suggesting its use could involve multiplexing of CRISPR targets (Port *et al*., 2020a). It is likely that more of these nucleases will be developed in the future to broaden CRISPR-Cas applications.

7.2.2 Delivery of Cas9–gRNA complexes

Protocols for genome editing with CRISPR/Cas9 have been extensively developed in *Drosophila* and later adapted for other insects (Kondo and Ueda, 2013; Ren *et al*., 2013, 2014; Yu *et al*., 2013; Bassett and Liu, 2014a,b). In *Drosophila*, a variety of delivery mechanisms for Cas9 and gRNA have been used, such as plasmid DNA, mRNA, protein and transgenic expression. Initially, experiments used two plasmids, encoding either Cas9 or gRNA under the control of a strong promoter such as *Heat Shock Protein 70* (*Hsp70*) or U6, respectively. As a result of this effort, a large number of plasmid constructs for either component of CRISPR/Cas9 were made available in the Addgene plasmid repository (https://www.addgene.org/crispr) (accessed 5 April 2022). Co-microinjection of both plasmids into pre-blastoderm embryos resulted in successful mutagenesis, but with relatively low efficiency of mutant phenotypes and germline transmission (Gratz *et al*., 2013). Alternatively, both components for CRISPR/Cas9 can be supplied as an RNA mix, where Cas9 mRNA and the gRNA can be transcribed *in vitro* in the laboratory using plasmid DNA or a PCR product as a template (Bassett *et al*., 2013; Yu *et al*., 2013). This strategy is highly efficient in *Drosophila*, resulting in high frequencies of mutant phenotypes and germline mutations. Probably, using RNA instead of a plasmid DNA facilitates targeting somatic embryonic cells and germline cells sooner in development due to the delay inherent in the transcription of Cas9 DNA, translation and assembly of Cas9–gRNA complexes. However, in non-model insects, such as screwworms and butterflies, the efficiency of this method is much lower than in *Drosophila*.

For many non-model insects, the highest efficiencies of CRISPR insertions/deletions (indels) are obtained using a mix of Cas9 protein and *in vitro* transcribed gRNA (Li *et al*., 2017; Zhang and Reed, 2017; Paulo *et al*., 2019). Indeed, Cas9 protein can be made in the laboratory from bacterial expression vectors or bought commercially

from a variety of providers. In our hands, the best concentration ratio is 500 µg Cas9 protein/µl and 300 µg gRNA/µl (Concha *et al.*, 2019; Paulo *et al.*, 2019, 2021), but for each insect species it is recommended to test different combinations ranging from 250 to 600 µg of each component/µl. Generally, in insects the main method for the delivery of the CRISPR/Cas9 components is microinjection into embryos. Although standard commercial needles are regularly used for this purpose for *Drosophila*, for other insects it is highly recommended to fabricate custom-made needles using a needle puller and beveller, which allows for a great deal of variation in the shape of tappers that can be better adapted for each particular species (Miller *et al.*, 2002). Some microinjection protocols use colour dyes in their injection mixes to help visualize the mix diffusing along the embryo during injection, which may be useful as an indicator of good injection procedure and when working with long-shaped embryos in which injected components may diffuse only partially (Erickson *et al.*, 2016).

An alternative method for delivering Cas9 and gRNA to embryos is by transgenic expression. In the case of *Drosophila* this appears to be the most efficient method of delivery (Kondo and Ueda, 2013; Ren *et al.*, 2013; Sebo *et al.*, 2014). Several research groups developed transgenic lines expressing Cas9 under the control of promoters or enhancers to influence when and where it is expressed (Port *et al.*, 2014). A number of lines have been created using either strong constitutive promoters like *actin5C,* which induce the expression of Cas9 ubiquitously, or germline promoters such as *vasa* and *nos* for the expression of Cas9 in the ovaries and testes, which was shown to be very efficient in generating germline mutants (Baena-Lopez *et al.*, 2013; Ren *et al.*, 2013; Port *et al.*, 2014). One study created two separate transgenic strains expressing nos-Cas9 and U6-gRNA, respectively, and then crossed them to each other to generate a transgenic strain expressing both components (Kondo and Ueda, 2013). Such a strain generated dsDNA breaks at the desired target site with very high efficiency and also showed a high efficiency of germline transmission of the mutations. Another study used a single component system, creating transgenic strains carrying a vasa-Cas9 construct that expresses Cas9 in the germline followed by microinjecting U6-gRNA plasmids into pre-blastoderm embryos of this strain (Sebo *et al.*, 2014). This method showed improved survival rates of injected embryos and an excellent efficiency of germline transmission. However, mutants exhibited low fertility, likely due to off-target effects in the germline. Generally, in these transgenic expression systems, after obtaining the desired mutant strains, the CRISPR components are removed by outcrossing. More recently, researchers developed transgenic temperature-inducible lines of the fruit fly *Drosophila suzukii*, which carry a *Dmhsp70-Cas9* construct (Yan *et al.*, 2021). In these lines Cas9 is expressed endogenously by the insects under the control of the heat-inducible *D. melanogaster hsp70* promoter. Upon microinjection of sgRNA directed against the pigment gene *yellow* into pre-blastoderm embryos, this system results in efficient somatic and germline mutations that give rise to the *yellow* phenotype. Furthermore, the expression of Cas9 can be greatly increased by induction with heat shock, giving the system more flexibility to increase expression as desired. Alternatively, the UAS-Gal4 system has been used to generate tissue-specific knockouts by expressing Cas9 in a tissue-specific manner (Xue *et al.*, 2014). This system showed very high efficiency of mutagenesis and has been tested extensively in *Drosophila,* where there is even a library of strains containing different U6-gRNA constructs and improved UAS-Cas9 constructs, which are ready to use in crossing experiments (Port *et al.*, 2020b).

In non-model insects these transgenic systems may be harder to implement due to fewer species-specific molecular tools, lower ease of rearing, lower egg availability, longer developmental cycles, greater space requirements and other considerations. However, a few of these systems have been established in insects of economic relevance. In silkworms, which are economically and culturally relevant

due to their silk production, CRISPR/Cas9 transgenic strains have been created to disrupt genes of pathogenic viruses and fight fungal infections (Dong *et al*., 2018, 2019; Zhang *et al*., 2019) (see Sezutsu and Tamura, Chapter 20, this volume). In mosquitoes, which are important as human-disease vectors, several transgenic systems based on the nos-Cas9 and U6-gRNA components have been created for the development of gene drives aimed at population control in the field (Gantz *et al*., 2015; Hammond *et al*., 2016; Simoni *et al*., 2020) (see Arien *et al*., Chapter 10; Bottino-Rojas and James, Chapter 11, this volume).

7.2.3   Identifying genomic modifications

CRISPR technology was initially developed in insects creating loss-of-function mutations in genes with a cell-autonomous visible phenotype, like eye colour or body pigmentation, such that the resulting mutants could be easily recognized. Mutant G_0 insects present mosaic genomic modifications and phenotypes. This means that only some cells are successfully targeted while others remain unmodified, creating an array of different mutations at the target site. This is explained by the methodology used: where Cas9–gRNA complexes are microinjected into pre-blastoderm embryos, they diffuse along the embryo to reach the different syncytial nuclei at varying concentrations, creating different mutations in each one. Many cells will be modified but some will have small indels and others will have large indels at the target site. This fact has some implications for the kind of molecular analysis performed to evaluate the genomic mutations. Genotyping can be carried out by PCR, amplifying a genomic region that spans the CRISPR/Cas9 target site, followed by either Illumina sequencing or cloning of the PCR products and Sanger sequencing of each individual clone. The detection of indels in the sequences can be performed using a variety of software (Bennett *et al*., 2020), such as Tracking of Indels by Decomposition (TIDE) (https://tide.nki.nl/, accessed 5 April 2022) or the Synthego Inference of CRISPR (ICE) tool (https://www.synthego.com/products/bioinformatics/crispr-analysis, accessed 5 April 2022). This will reveal the sequences of a range of different mutations across the target site.

For quickly identifying CRISPR G_0 mutants that do not have a visible phenotype, either because the mutant phenotype is cell lethal or because the target gene does not code for a morphological feature, two methods are commonly used: high resolution melt analysis (HRMA) (Housden and Perrimon, 2016) and T7 endonuclease I assay (Mashal *et al*., 1995). Both methods rely on the isolation of genomic DNA from mutant insects and amplification by PCR of a genomic region spanning the target site, where mosaic mutants or heterozygous mutants are identified by the presence of mismatches in sequence of the annealed PCR products caused by the diversity of mutations present. HRMA is performed as a real-time PCR assay in which the melting temperature (detected by the thermal cycler) of mismatched fragments varies from that of wild-type DNA. In T7 endonuclease I assay, the enzyme will cut mismatched DNA fragments while leaving wild-type matching DNA fragments intact. Both techniques take only a few hours to perform, allowing for a quick screening of induced mutations. A disadvantage of these methods is that, in the case of the presence of SNPs in the target sequence, this may induce false positives. A solution to this problem is a better genomic characterization of the target obtained by sequencing the targeted region in several individuals of the same species before designing the CRISPR/Cas9 experiment (see section 7.2.1, above).

Another method that is often used is the heteroduplex mobility assay (HMA) in which the PCR products are boiled and then cooled very slowly to favour the formation of heteroduplexes (Foster *et al*., 2019). The PCR fragments then migrate in PAGE electrophoresis, revealing differences between the mutant and wild-type samples. This method is simpler and cheaper than the previous alternatives since it does not require expensive enzymes or special laboratory equipment. One challenge for assessing mutations is

genotyping insects without killing them or affecting their ability to reproduce. In butterflies, protocols exist where samples are taken from larvae spines and used for DNA extraction without harming the insects and, in this way, it is possible to genotype them before they develop to adults, reducing the number of individuals reared (Markert *et al.*, 2016). In our hands, we developed a protocol for genotyping adult mutants by removing a leg from each G_0 adult after crossing and females had laid eggs, allowing us to continue rearing only the progeny of crosses with mutant G_0 adults (Paulo *et al.*, 2019, 2021).

While much can be learned about gene function from mutant G_0 insects, it may be desirable to breed a strain that is homozygous for the targeted mutation. In this case, the CRISPR-Cas9 *in vitro* essay is a very efficient method to distinguish heterozygous from homozygous germline mutants (Kim *et al.*, 2014). In this assay, a PCR product spanning the genomic mutation is incubated *in vitro* with Cas9–gRNA complexes. Only wild-type DNA fragments or heterozygous mismatched PCR products will be cleaved, while PCR products with homozygous mutations will remain intact, as the cleavage site for Cas9 will have been removed by the mutation. This strategy is particularly useful for making homozygous strains, because it allows the selection of the homozygous individuals for inbreeding.

7.3 Applications of CRISPR/Cas9 in Insects

Since its publication nearly a decade ago, CRISPR/Cas9 genome editing has been established for many insect species and is now routinely used for making site-directed indels. From an applied perspective, genome editing can help to create more sustainable and environmentally friendly methods for the control of insects of economic and public health importance. To date, the main applications of genome editing in insects have been in developing markers for mutants and testing the function of potential targets for creating gene drives. On the other hand,

functional genomics is a powerful tool for the investigation of gene function in model and non-model organisms, to help understand basic research questions in physiology, development and evolution. We discuss a few examples of these applications below.

7.3.1 Developing markers for mutants

In non-model insects, the most common markers for genome editing are eye and body coloration genes. Indeed, these were the first targets to be tested, because they generate visible phenotypes that also serve as a proof-of-principle for the CRISPR/Cas9 technique and may be used as targets of insertion in the construction of gene drives. One of the most conserved and frequently used eye-colour genes in insects is *white*, which codes for an ABC transporter involved in the movement of red and brown eye-colour pigment precursors, guanine and tryptophan, into the developing eyes during pupation (Mackenzie *et al.*, 1999). The disruption of *white* results in white eye colour that can be rescued by the introduction of a wild-type *white* allele, restoring the wild-type dark red eye colour. Thus, a white mutant line may be a useful marker for genomic insertion of an engineered cassette containing a wild-type *white* gene. CRISPR *white* mutants have been created in many Diptera, including *D. suzukii* (Li and Scott, 2016), *Drosophila subobscura* (Tanaka *et al.*, 2016), *Ceratitis capitata* (Meccariello *et al.*, 2017), *Bactrocera oleae* (Meccariello *et al.*, 2020), *Anastrepha ludens* (Sim *et al.*, 2019), and some Lepidoptera (Khan *et al.*, 2017; Liu *et al.*, 2021). However, in *D. melanogaster* and *D. suzukii*, disruption of *white* has been associated with decreased mating behaviour, which suggests caution for its use as an integration marker in these species (Xiao *et al.*, 2017; Yan *et al.*, 2020). Other eye pigment pathway genes, like *kynurenine 3-hydroxylase* (Liu *et al.*, 2019), *scarlet* (Koidou *et al.*, 2020; Liu *et al.*, 2021) and *cardinal* (Xu *et al.*, 2020), have also been tested as markers in many insects and a few have been successfully used in the construction of gene drives (Gantz *et al.*, 2015; Carballar-Lejarazú *et al.*, 2020).

Interestingly, the pupal pigmentation gene *white pupa* (*wp*) has recently been identified in three tephritid species as an MFS transporter that likely transfers catecholamines from the haemolymph to the pupal cuticle (Ward *et al*., 2021). CRISPR knockout of this gene results in loss of pigmentation in the pupae, giving rise to the *white pupa* phenotype. This mutation had been used for decades as a marker in medfly genetic sexing strains (GSS) employed in pest control programmes using the sterile insect technique (SIT), but the gene involved was unknown until now. Given that this gene is very conserved in many insect species, including agricultural pests and mosquito disease vectors, it may be a potentially reliable marker for other insects used in SIT programmes. Similarly, genes involved in body pigmentation are also potential markers for genome editing and have been tested frequently in non-model insects. For example, the *yellow* gene in housefly (Heinze *et al*., 2017), in the Australian sheep blowfly *Lucilia cuprina* and in the New World screwworm *Cochliomyia hominivorax*, the latter two being major livestock pests (Paulo *et al*., 2019), is required for normal melanization of the body cuticle and its disruption with genome editing results in a brown body phenotype (*bwb*). This phenotype, similar to the eye-colour gene *white*, is homozygous recessive, which requires that both autosomal alleles for the *yellow* gene are disrupted to give a visible phenotype (Fig. 7.2). Therefore, a mutant *bwb* line could be used as a marker for site-directed integration of gene cassettes including the wild type *yellow* gene, which would restore wild-type body pigmentation upon integration. Yellow CRISPR mutants have
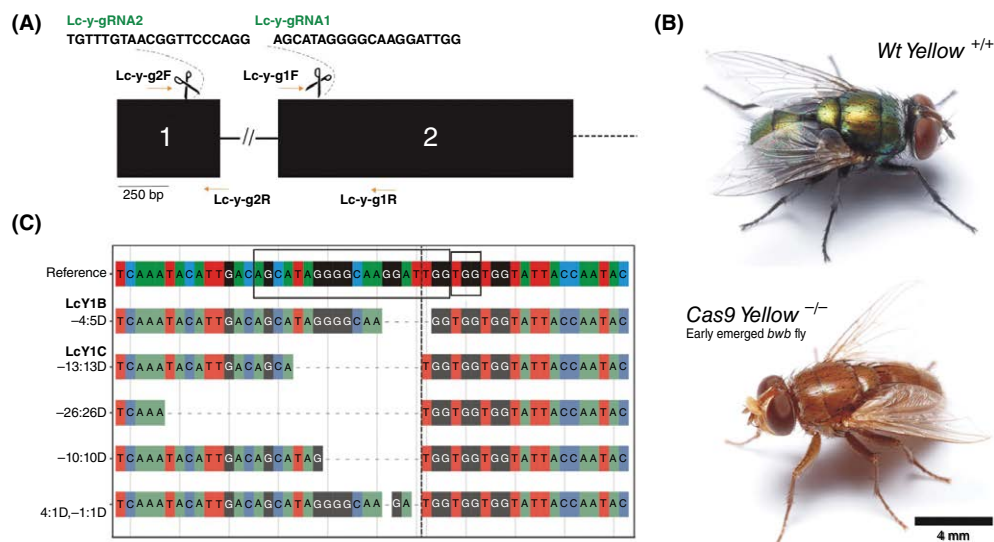
Fig. 7.2. Genome editing of the *yellow* gene in the sheep blowfly *Lucilia cuprina*. (A) The *Lucilia cuprina yellow (Lc-y)* gene was targeted using two sgRNA designed against the first and second exons of the gene. The sgRNAs are shown in green letters, the cutting sites are shown by black scissors and the primers used for genotyping are shown as orange arrows. **(B)** Only Lc-y-RNA1 was efficient in generating indels, which are shown in a multiple sequence alignment against the wild-type *Lc-y* sequence. The targeted site is shown as a rectangular box, the PAM is shown as a square box after the target sequence, and the predicted cut site is marked with a vertical dashed line. Two homozygous lines, *bwb*-mutant strains LcY1B and LcY1C, were established after crossing back to the parental wild-type strain, and their mutant alleles are shown in the alignment. **(C)** Comparison between the wild type (above) and the *yellow* homozygous knockout (below) phenotypes of *L. cuprina*. From Paulo *et al*. (2019).

also been created in other dipterans like *D. suzukii* (Yan *et al.*, 2021) and in Hemiptera (Nie *et al.*, 2021), Lepidoptera (Chen *et al.*, 2018; X.-L. Liu *et al.*, 2020b; Wang *et al.*, 2020) and mosquitoes (Liu *et al.*, 2019). Another pigmentation gene, *ebony*, is also required for proper melanization of the body cuticle in insects and has been commonly used as a marker (Bi *et al.*, 2019). In contrast to *yellow*, mutants for this gene show darker body pigmentation. In silkworms, a series of genes involved in larval cuticle pigmentation have been isolated and functionally evaluated using CRISPR targeting, such as *teashirt*, *tiptop*, *BLOS2* and *orcokinin* (Zhu *et al.*, 2017; P. Wang *et al.*, 2019a; Zhang *et al.*, 2020), showing promise as potential markers for genetically modified strains.

Genetically modified insects that are suitable for a field control programme require a marker that distinguishes the released insects when they are caught in traps during field monitoring. Furthermore, it would be desirable to assess if wild females caught in traps were mated to wild-type or genetically modified released males. From this point of view, fluorescent protein marker genes, like the ones regularly used for transgenesis experiments, are also very useful markers for CRISPR 'knock-in' experiments in genetic pest control strategies such as gene drives (Gantz *et al.*, 2015; Simoni *et al.*, 2020) or precision-guided SIT (see Raban and Akbari, Chapter 8, this volume). Integration cassettes containing a fluorescent marker under the control of a tissue-specific promoter, like the eye-specific promoter *3xP3* and the testes-specific promoter *beta-2-tubulin*, or a strong ubiquitous promoter, like *polyUbiquitin* (*pUb*), have been successfully used in fruit flies (Li and Handler, 2017; Aumann *et al.*, 2018) and mosquitoes (Kistler *et al.*, 2015). Furthermore, a *D. suzukii* transgenic strain was developed carrying a sperm-specific fluorescent marker, *beta-2-tubulin-DsRed*, which showed strong red fluorescent expression in male pupae, male adults and female spermathecae. This marker could potentially be used for the identification of wild female insects mated to genetically modified males released in the field (Ahmed *et al.*, 2019).

7.3.2 Testing gene function before making a gene drive

Gene drives are selfish genetic elements capable of skewing their own inheritance ratio to a supra-Mendelian rate and, consequently, driving themselves to spread quickly through a wild population despite conferring no fitness benefit on individuals that carry them. Many of these selfish genetic elements have been identified in microorganisms and Metazoans (Werren *et al.*, 1988; Burt and Trivers, 2006). Indeed, in the early 2000s they inspired the design of synthetic strategies for pest insect population control using homing endonuclease genes (HEGs) (Burt, 2003) and, more recently, CRISPR/Cas9 (Gantz and Bier, 2015; see Raban and Akbari, Chapter 8; Champer, Chapter 9, this volume).

To suppress an insect pest population, a synthetic gene drive can be created to bias the sex ratio in a population such that one of the sexes is reduced and eventually eliminated, thus causing the population to crash. In order to create such a gene drive, the identification of genes involved in the sex determination pathways of specific insect species is very useful. A major regulatory sex determination gene could be targeted with CRISPR/Cas9 to disrupt its function and, hence, bias sexual development towards a single-sex progeny. To develop this, it is essential to test different targeting sites in each candidate gene to assess the efficiency of gene disruption and sexual transformation with each one. If these preliminary experiments are successful, selected targeting sites within chosen genes can be used in the construction of a gene drive. Similarly, other components of the gene drive, such as species-specific promoters, must also be tested to make sure they are efficient, as insect gene promoters are often only active in the same species or in a near relative.

In dipteran insects, sex determination pathways are very diverse in their upstream master sex-determining signals while the middle and downstream regulators have remained very conserved (Bopp *et al.*, 2014) (see Arien *et al.*, Chapter 10, this volume). In the fruit fly *D. melanogaster*, sex determination consists of

a genetic regulatory hierarchy in females that begins with the activation of the *Sex-lethal* gene (*Sxl*), promoting female development through a short cascade of downstream genes. SXL regulates the splicing of *transformer* pre-mRNA (*tra*) such that only females produce an RNA that codes for a full-length and functional TRA protein (Belote *et al.*, 1989; Sosnowski *et al.*, 1989). TRA forms a complex with TRA2, a cofactor that is constitutively expressed in both sexes, and promotes the female-specific splicing of *doublesex* pre-mRNA (*dsx*), the last component of the regulatory hierarchy. In the absence of functional SXL protein, male-specific splicing of *tra* occurs by default, resulting in the development of the male phenotype. Based on this knowledge, it has been possible to isolate homologous sex-determining genes in *D. suzukii*, an invasive agricultural pest species. CRISPR/Cas9 targeted sequence disruption of the *DsSxl* gene results in mosaic masculinization of female genitalia and reproductive tissues, suggesting that this gene could be a good target for creating a gene drive in this species (Li and Scott, 2016). In another study, *D. suzukii* mutants were created by CRISPR-Cas9 HDR, with a cassette carrying a temperature-sensitive loss-of-function point mutation for *tra2* (Li and Handler, 2019). The mutants develop as normal and fertile insects at permissive temperatures below 20°C, but at restrictive temperatures of 29°C, XX individuals develop as sterile intersexes with a predominant male phenotype while XY individuals develop as normal males but are sterile. In this case, generating a specific temperature-sensitive mutation in *Dstra2* could be useful for developing male sexing strains for SIT, as it adds a conditional activation for the loss-of-function phenotype while conserving a means for rearing the insects at the permissive condition. However, the strong sterility observed in mutant males and females would have a fitness cost too elevated to be useful in the creation of a gene drive, preventing the gene construct from spreading in a population. Similarly, other researchers used *D. melanogaster* as a model to study the feasibility of creating a gene drive using the *tra* gene as a homing gene target for CRISPR/Cas9 (Carrami *et al.*, 2018). The study showed that disruption

of *tra* in insects carrying the gene drive transformed females into males, but the occurrence of NHEJ events in some of the progeny increased the rate of mutagenesis at the target site, resulting in frequent in-frame drive-resistant alleles that compromised drive efficiency. This prompted the authors to use the data from these experiments to perform a simulation of a gene drive based in the homing of the *tra* gene in *C. capitata* and found that, by using at least four gRNA to target *tra*, the frequency of in-frame NHEJ events at the target site should be greatly reduced and the efficiency of the drive system improved.

In tephritid fruit flies, which are major agricultural pests, the *Sxl* gene is not involved in sex determination (Bopp *et al.*, 2014). Instead, a Y chromosome-linked male determining gene, *Maleness on the Y* (*MoY*), is the primary masculine sex-determining signal (Meccariello *et al.*, 2019). MOY suppresses female-specific *tra* splicing, thus favouring the expression of male-specific *tra* transcripts to establish the male phenotype. In the absence of MOY in XX embryos, a maternal contribution of *tra* activates its own female-specific splicing in a positive feedback loop, leading to female development. Interestingly, CRISPR/Cas9 targeting of *MoY* in *C. capitata* results in sexual transformation of males into fertile females and intersexes, while overexpression of *MoY* in XX embryos induces masculinization. These findings suggest that *MoY* is a good potential candidate gene for creating a male-converting gene drive. In contrast, in another study, CRISPR/Cas9 targeting of *tra* in *C. capitata* resulted in a full phenotypic transformation of females into males with activation of male-specific splicing of *tra* mRNA (Primo *et al.*, 2020). However, the phenotypic XX males rarely presented mutations at the targeted site, suggesting that the establishment of *Cctra* female-specific autoregulatory loop was prevented by Cas9–gRNA binding to the target site rather than by cleavage. This hypothesis was further supported by the observation that dCas9, an inactive form of Cas9, induced a partial female-to-male reversal of the genitalia and of *Cctra* splicing. In this study, the chosen gRNA for *Cctra* targeting would be inadequate for creating a

male-converting gene drive, due to the low rate of mutagenesis obtained.

In the housefly *Musca domestica*, the primary signal of the sex determination cascade is, like in tephritids, a male-determining factor (M-factor), which can reside on any of the chromosomes. This M-factor was found in chromosomes Y, II, III or V and named *Musca domestica male determiner (Mdmd)*. Targeted disruption of *Mdmd* using CRISPR/Cas9 results in complete sex reversal to fertile females, because of a shift from male to female splicing of the downstream genes *transformer* and *doublesex*. The presence of *Mdmd* in different chromosomes suggests that the gene arose from a duplication event and was then repurposed as an essential splicing regulator (Sharma *et al.*, 2017). Indeed, these comparisons show that the primary signals in insect sex determination can be very diverse across the Diptera.

Taken together, these examples illustrate the importance of extensive testing of efficient targeting sites within potential homing genes as preliminary work in the creation of gene drives. Similar experiments have been conducted in other fruit flies (Zhao *et al.*, 2019; Peng *et al.*, 2020), blowflies (Paulo *et al.*, 2019), mosquitoes (Hall *et al.*, 2015; Kyrou *et al.*, 2018; P. Liu *et al.*, 2020a) and Lepidoptera (Chen *et al.*, 2019; Du *et al.*, 2019; Y.-H. Wang *et al.*, 2019b; Wang *et al.*, 2020) with the aim of selecting components that may be used in insect genetic population control strategies.

7.3.3 Functional genomics in evolution

Basic research has also experienced an explosion of studies using genome editing to interrogate the function of genes involved in a variety of processes, from physiology to behaviour. In evolutionary biology, CRISPR/Cas9 has emerged as a powerful tool for site-specific genomic modification of genes that are considered 'evolutionary hotspots', genes that have been repeatedly targeted by natural selection to produce variation, and for investigating the mechanisms

involved in their regulation (reviewed in Mc-Millan *et al.*, 2020).

Butterflies are one of the best-explored study systems where CRISPR/Cas9 has been used to test the function of genes and regulatory elements, mainly involved in wing-colour pattern development. Butterfly diversity has offered the benchmark to test functional genomic hypotheses, including the repeatability of evolution (Concha *et al.*, 2019). Adaptive radiations are a particular case in the diversification of butterflies and present clear opportunities to study the genetic and developmental mechanisms that underlie the evolution of novel morphologies. *Heliconius* butterflies display one of the most visually diverse radiations in the animal kingdom, with over 40 species that rapidly diversified in the Neotropics within the past 12 million years, including repeated cases of wing-pattern mimicry between distantly related species (Merrill *et al.*, 2015). These butterflies are well known for their colourful wing patterns, which are used for warning their predators that they are toxic and as conspecific mating signals (Benson, 1972; Chouteau *et al.*, 2017; Merrill *et al.*, 2019). With the arrival of modern genomic technologies, the identity of four major genes, *WntA*, *cortex*, *optix* and *Aristaless1*, involved in modulating pattern variation across the genus was revealed (Reed *et al.*, 2011; Martin *et al.*, 2012; Nadeau *et al.*, 2016; Westerman *et al.*, 2018). We focus this last section on *Heliconius* butterflies, since they have provided clear evidence on the power of CRISPR/Cas9 to test evolutionary hypotheses in a system that is not a model organism.

The discovery of the 'wing patterning' toolkit in *Heliconius* has allowed the formulation of broad general conclusions about how morphological diversity is generated. Foremost, patterning loci identified in *Heliconius* have also been shown to affect pattern variation broadly across butterflies and moths. For example, the gene *cortex* underlies variation in white, yellow, orange and black pattern elements in *Heliconius* (Nadeau *et al.*, 2016) but also affects colour pattern in the peppered moth *Biston betularia* (Van't Hof *et al.*, 2016) and other geometrids, and the silkworm *Bombyx mori*
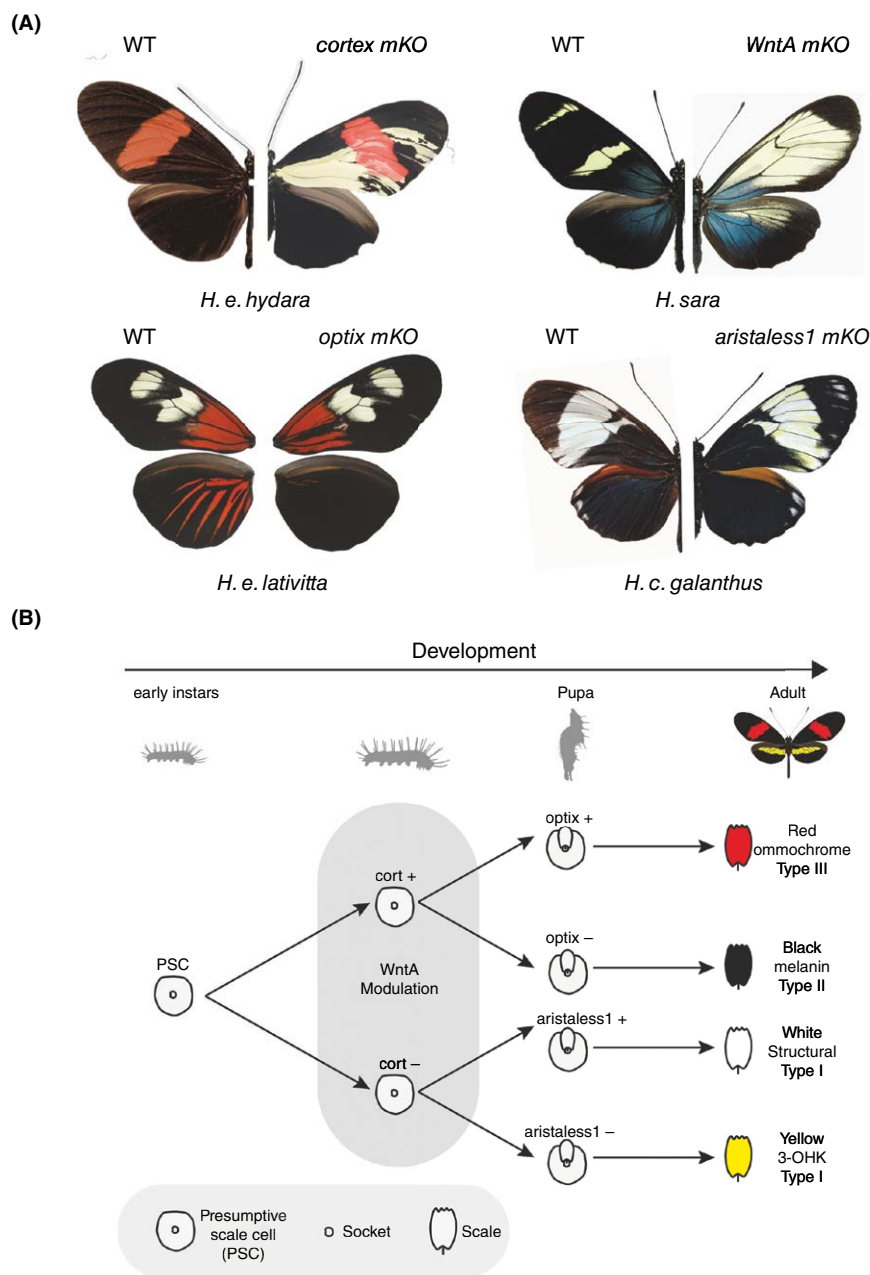
Fig. 7.3. Functional knockouts of *Heliconius* butterfly colour patterning genes. (A) CRISPR/Cas9 genome editing of the major colour patterning genes in *Heliconius*, which are responsible for most of the colour pattern variation in the genus. Functional disruption of *cortex* in *H. erato hydara* results in the transformation of black and red wing scales into yellow or white scales across the whole surface of the wing. Gene knockout of *WntA* in *H. sara* results in a shift of colour pattern boundaries, with loss of black patterns and an extension of yellow pattern elements towards the base of the forewing and the anterior border of the hindwing. Loss-of-function mutations in *optix* result in a switch of scale cell colour from red to black in *H. e. lativitta*. Disruption of the *aristaless1* gene in *H. cydno galanthus* results in a colour switch

(Joron *et al.*, 2006; Ito *et al.*, 2016; Van't Hof *et al.*, 2016, 2019; VanKuren *et al.*, 2019). Secondly, the majority of these patterning genes' protein sequences are very conserved and are used extensively throughout development, and later simply redeployed on a developing wing to affect pattern variation. The transcription factor *optix*, for example, was first discovered in *Drosophila*, where it has a role in eye development and is also expressed in the developing wing and haltere primordia, where it is involved in vein patterning (Seimiya and Gehring, 2000; Organista *et al.*, 2015). In *Heliconius* butterflies, *optix* is expressed in the optic lobe (Martin *et al.*, 2014), where it presumably plays a role in neural development, but has also evolved new expression domains in the developing pupal wing, where its expression perfectly prefigures red pattern variation on adult butterfly wings (Al Khatib *et al.*, 2017; Reed *et al.*, 2011; Monteiro, 2012). Finally, pattern variation is created mostly by regulating when and where these genes are expressed during development. The gene *WntA* is a member of the Wnt family of signalling ligands and has, similarly, evolved new patterning roles, with dramatic shifts in *WntA* expression driving pattern variation in *Heliconius* (Martin *et al.*, 2012) and other nymphalid butterflies (Gallant *et al.*, 2014; Martin and Reed, 2014).

With the recent development of CRISPR/Cas9 technology in *Heliconius*, researchers have been able to perform functional assays by inducing targeted mutations in the genes' coding sequence, as well as making deletions in putative regulatory elements, in order to observe the resulting mutant phenotypes (reviewed in Livraghi *et al.*, 2018). This work has led to substantial insights on the function of major switch genes in pattern variation (Mazo-Vargas *et al.*, 2017; Zhang *et al.*, 2017; Westerman *et al.*, 2018; Concha *et al.*, 2019; Livraghi *et al.*, 2021). Functional experiments have been done by evaluating mutant G_0 butterflies, as the targeted genes are required for other developmental processes and the efficiency of mutagenesis is high enough, allowing us to see striking mosaic phenotypes in the adult butterflies (Fig. 7.3A). Indeed, functional studies suggest that *WntA* and *cortex* are expressed early in wing development and act to establish positional information for the downstream expression of selector genes that determine wing-scale cell colour (Fig. 7.3B). CRISPR knockout (KO) of *WntA* results in the loss of black pattern elements and a shift in colour pattern boundaries in *Heliconius* wings, where black wing scales are transformed to either red, yellow or white scales, and in some cases also from colour to black scales, depending on the genetic background (Concha *et al.*, 2019). Furthermore, functional knockout of *WntA* in co-mimetic *Heliconius* species has revealed a considerable divergence in gene regulatory networks between co-mimics. The novel understanding obtained with CRISPR/Cas9 functional work on wing pattern development across *Heliconius* indicates that the genetic and developmental mechanisms of evolution may be less predictable than once thought (Van Belleghem *et al.*, 2021). CRISPR knockout of *cortex* results in the transformation of wing scales to yellow or white colour scales across the whole wing, regardless of the shape of colour pattern boundaries (Livraghi *et al.*, 2021). Functional disruption of *optix* and *Aristaless1*, two transcription factors that are expressed

Fig. 7.3. Continued.

from white to yellow colour pattern elements. **(B)** Proposed model of interactions among colour patterning loci. During wing development, scales express key effector genes that lead to alternative scale cell types. Early presumptive scale cells (PSCs) express cell specification genes such as cortex, which initiate differentiation into Type II (optix−) or Type III (optix+) scales. In the absence of *cortex*, scale cells differentiate into Type I scales, which differ in pigmentation state based on 3-hydroxylkynurenine synthesis controlled by *aristaless1* expression. Under this model, WntA acts as a landscape modifier, whereby Wnt signalling modifies the *trans* environment experienced by differentiating scale cells, in turn delineating the boundaries in which specific differentiation factors can act. Images from Concha *et al.* (2019), Livraghi *et al.* (2021), Westerman *et al.* (2018) and Zhang *et al.* (2019). Model from McMillan *et al.* (2020).

later, during pupal wing development, result in binary shifts in scale cell colour, from red to black and from white to yellow, respectively, suggesting that these genes function as genetic colour switches (Zhang *et al*., 2017; Westerman *et al*., 2018).

Under a simple model (Fig. 7.3B), *cortex* acts as a 'master' regulator of scale-cell identity, where a *cortex* positive state initiates a differentiation cue that sets up a permissive environment for either melanization (*optix* negative) or red pigment synthesis (*optix* positive), consistent with a role in switching between ommochrome and melanin synthesis. In contrast, *cortex* negative cells become either yellow or white depending on the expression of *aristaless1*, which represses the pathway leading to the synthesis and deposition of the yellow 3-OHK pigment (Westerman *et al*., 2018). If *cortex* is acting as a master 'on-off' switch among scale cell types, the signalling ligand *WntA* might be best conceptualized as modifying the overall regulatory landscape used to provide the positional information for the establishment of boundaries between scale-cell types. In contrast to *cortex*, *WntA* effects are limited to specific wing pattern elements and the extent of pattern induction across the wing varies both within and between species. In summary, wing pattern diversity in *Heliconius* butterflies may be understood as a developmental process that results from the genetic regulation of wing scale-cell specification and differentiation.

One idea to emerge from developmental biology is that 'hotspot genes' are often associated with complex *cis*-regulatory variation (Stern and Orgogozo, 2009). This is certainly true of the major patterning loci identified in *Heliconius*. Expression and association studies have revealed that *cis*-regulatory variation is important in driving wing patterning diversity, and a few studies suggest that colour pattern variation is controlled by modular *cis*-regulatory elements (CREs) (Wallbank *et al*., 2016; Enciso-Romero *et al*., 2017; Van Belleghem *et al*., 2017). Consistent with this perspective, CRISPR/Cas9 excision of potential CREs, identified using a combination of epigenetic profiling and genotype × phenotype analysis, around

WntA and *cortex*, resulted in the appearance of discrete colour pattern elements (Livraghi *et al*., 2021; Concha *et al*., 2022 unpublished results) (Fig. 7.4). This evidence is coherent with the idea that new wing pattern phenotypes can evolve rapidly by simply reshuffling these discrete regulatory regions. However, similar work on putative CREs identified near *optix* resulted in highly pleiotropic mutant phenotypes that affected several pattern elements simultaneously, suggesting that more constraints are acting at colour pattern loci than previously hypothesized (Lewis *et al*., 2019). Overall, these recent functional experiments have begun to decipher the underlying regulatory networks controlling wing colour patterns and how they may have evolved. From these results emerges a complex evolutionary story of many interacting loci and partly independent genetic architectures underlying convergent evolution.

7.4   Concluding Remarks

The advent of site-directed genome editing technology has greatly improved the horizons of scientific research generally and had an important impact in insect physiology, genetics and applied molecular biology. In approaching basic research questions, it is now possible to edit the genome of non-model insects, which had been very difficult to interrogate without proper transgenesis or molecular genetic tools. Indeed, we can now perform loss-of-function studies to test specific hypotheses about the roles of genes and, together with other emerging genomic technologies (such as whole genome sequencing, ATAC-seq, RNA-seq) (Wang *et al*., 2009; Buenrostro *et al*., 2015; Paula, 2021), we can begin to unravel the molecular and developmental mechanisms underlying physiological, behavioural and evolutionary processes.

Despite this progress, technological gaps still remain. For example, methods for performing site-directed insertions in the genomes of insects have been poorly developed, with protocols available in very few
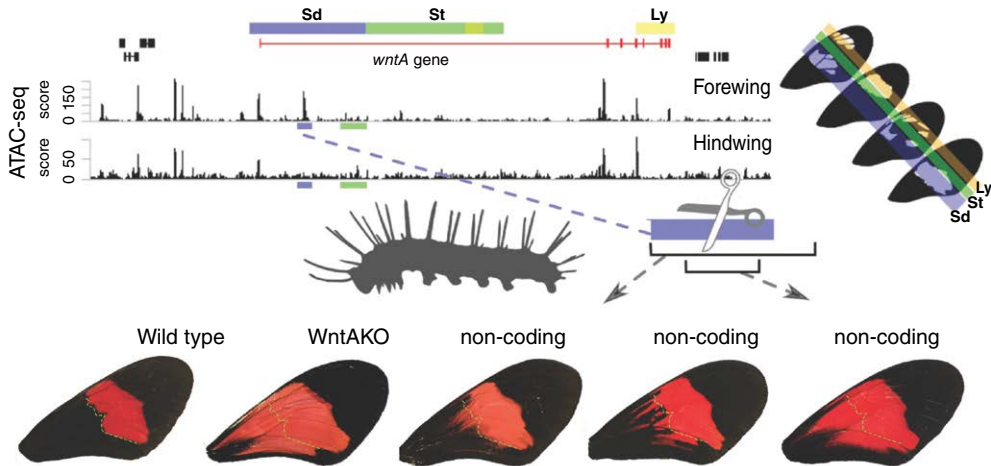
Fig. 7.4. *Cis*-regulation of the *WntA* gene in *H. erato* is revealed by CRISPR/Cas9 targeted excisions of putative *cis*-regulatory elements (CREs). A combination of association studies and chromatin accessibility using ATAC-seq has allowed the identification of regions of open chromatin upstream of the *WntA* gene with strong association with wing pattern variation. The *WntA* gene is shown in red, with exons represented by vertical lines and introns by horizontal lines. Genomic regions associated with colour pattern variation are shown as a blue rectangle (Sd region), green rectangle (St region) and yellow rectangle (Ly region), which relate to variation in specific areas of the forewing as shown in the top-left wing models. ATAC-seq results obtained from larval developing wings are shown underneath, correlated to the genomic regions described above. CRISPR/Cas9 excision of putative regulatory peaks containing CREs results in modular shifts in colour pattern consistent with the full *WntA* gene knockout phenotype (Carolina Concha, 2022, unpublished results).

non-model insect species and with low efficiencies of integration. There is still much work to be done in developing efficient methods for making 'knock-in' strains in insects and these will be fundamental in the future for proof of principle functional studies.

In developing strategies for insect pest control, CRISPR/Cas9 gene editing has provided a simpler and cleaner method for generating loss-of-function mutants, conditional mutants or for deleting genes entirely. In comparison with transgenesis, genome editing does not introduce exogenous transposase arms into the genomes of modified insects, which have a potential for being remobilized if they come in contact with endogenous transposases, posing a potential risk for their use in field releases. From this perspective, genome editing could help create efficient and safer sexing systems for field release, similar to the ones created by classical mutagenesis, comprising small sequence changes or SNPs that could also occur naturally and, therefore, be considered as a lesser risk to the environment than traditional transgenic strains. These 'cleaner strains' could be more appealing to regulators and local communities, increasing the likelihood that they may be approved for field releases.

Current research is now focused on creating gene drives for population control of agricultural insect pests and disease vectors. Some of these have been tested in *Drosophila* and in mosquitoes in large cages under laboratory conditions, showing much promise for efficient spread of desired genes in a population. Many researchers are now working on creating these systems for a variety of insect species of economic importance. However, this process takes time, due to the need for testing each of the components in each species of interest before creating the gene drive. Furthermore, many challenges to the application and safe use of these strains have appeared, including the evolution of resistance alleles that diminish drive efficiency, ensuring stability of the drive

cassette within the insect genome over time and containing the spread of the gene drive to selected populations in the field. Despite these difficulties, there are more tools now for engineering insect genomes than ever before and, with rapidly evolving modelling studies and molecular genetic designs, there will come a day when we have a variety of genetic strategies at our disposal for insect population control.

References

Ahmed, H.M.M., Hildebrand, L. and Wimmer, E.A. (2019) Improvement and use of CRISPR/Cas9 to engineer a sperm-marking strain for the invasive fruit pest *Drosophila suzukii*. *BMC Biotechnology* 19, 85. doi: 10.1186/s12896-019-0588-5

Al Khatib, A., Siomava, N., Iannini, A., Posnien, N. and Casares, F. (2017) Specific expression and function of the *Six3 optix* in *Drosophila* serially homologous organs. *Biology Open* 6, 1155–1164. doi: 10.1242/bio.023606

Aumann, R.A., Schetelig, M.F. and Häcker, I. (2018) Highly efficient genome editing by homology-directed repair using Cas9 protein in *Ceratitis capitata*. *Insect Biochemistry and Molecular Biology* 101, 85–93. doi: 10.1016/j.ibmb.2018.08.004

Baena-Lopez, L.A., Alexandre, C., Mitchell, A., Pasakarnis, L. and Vincent, J.-P. (2013) Accelerated homologous recombination and subsequent genome modification in *Drosophila*. *Development* 140, 4818–4825. doi: 10.1242/dev.100933

Barrangou, R. and Marraffini, L.A. (2014) CRISPR-Cas systems: Prokaryotes upgrade to adaptive immunity. *Molecular Cell* 54, 234–244. doi: 10.1016/j.molcel.2014.03.011.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P. *et al.* (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315, 1709–1712. doi: 10.1126/science.1138140

Bassett, A. and Liu, J.-L. (2014a) CRISPR/Cas9 mediated genome engineering in *Drosophila*. *Methods* 69, 128–136. doi: 10.1016/j.ymeth.2014.02.019

Bassett, A.R. and Liu, J.-L. (2014b) CRISPR/Cas9 and genome editing in *Drosophila*. *Journal of Genetics and Genomics* 41, 7–19. doi: 10.1016/j.jgg.2013.12.004

Bassett, A.R., Tibbit, C., Ponting, C.P. and Liu, J.-L. (2013) Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Reports* 4, 220–228. doi: 10.1016/j.celrep.2013.06.020

Belote, J.M., McKeown, M., Boggs, R.T., Ohkawa and R., Sosnowski, B.A. (1989) Molecular genetics of transformer, a genetic switch controlling sexual differentiation in *Drosophila*. *Developmental Genetics* 10, 143–154. doi: 10.1002/dvg.1020100304

Bennett, E.P., Petersen, B.L., Johansen, I.E., Niu, Y., Yang, Z. *et al.* (2020) INDEL detection, the 'Achilles heel' of precise genome editing: a survey of methods for accurate profiling of gene editing induced indels. *Nucleic Acids Research* 48, 11958–11981. doi: 10.1093/nar/gkaa975

Benson, W.W. (1972) Natural selection for Müllerian mimicry in *Heliconius erato* in Costa Rica. *Science* 176, 936–939. doi: 10.1126/science.176.4037.936

Bi, H.-L., Xu, J., He, L., Zhang, Y., Li, K. and Huang, Y.-P. (2019) CRISPR/Cas9-mediated *ebony* knockout results in puparium melanism in *Spodoptera litura*. *Insect Science* 26, 1011–1019. doi: 10.1111/1744-7917.12663

Bibikova, M., Golic, M., Golic, K.G. and Carroll, D. (2002) Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. *Genetics* 161, 1169–1175

Bibikova, M., Beumer, K., Trautman, J.K. and Carroll, D. (2003) Enhancing gene targeting with designed zinc finger nucleases. *Science* 300, 764. doi: 10.1126/science.1079512

Bier, E., Harrison, M.M., O'Connor-Giles, K.M. and Wildonger, J. (2018) Advances in engineering the fly genome with the CRISPR-Cas system. *Genetics* 208, 1–18. doi: 10.1534/genetics.117.1113

Bogdanove, A.J. and Voytas, D.F. (2011) TAL effectors: customizable proteins for DNA targeting. *Science* 333, 1843–1846. doi: 10.1126/science.1204094

Bolotin, A., Quinquis, B., Sorokin, A. and Ehrlich, S.D. (2005) Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology (Reading, England)* 151, 2551–2561. doi: 10.1099/mic.0.28048-0

Bopp, D., Saccone, G. and Beye, M. (2014) Sex determination in insects: variations on a common theme. *Sexual Development* 8, 20–28. doi: 10.1159/000356458

Buenrostro, J.D., Wu, B., Chang, H.Y. and Greenleaf, W.J. (2015) ATAC-seq: a method for assaying chromatin accessibility genome-wide. *Current Protocols in Molecular Biology* 109, 1–9. doi: 10.1002/0471142727.mb2129s109

Burt, A. (2003) Site-specific selfish genes as tools for the control and genetic engineering of natural populations. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 270, 921–928. doi: 10.1098/rspb.2002.2319

Burt, A. and Trivers, R. (2006) *Genes in Conflict: the Biology of Selfish Genetic Elements*. Belknap Press of Harvard University Press, Cambridge, Massachusetts.

Carballar-Lejarazú, R., Ogaugwu, C., Tushar, T., Kelsey, A., Pham, T.B. *et al.* (2020) Next-generation gene drive for population modification of the malaria vector mosquito, *Anopheles gambiae*. *Proceedings of the National Academy of Sciences USA* 117, 22805–22814. doi: 10.1073/pnas.2010214117

Carrami, E.M., Eckermann, K.N., Ahmed, H.M.M., Sánchez C, H.M., Dippel, S. *et al.* (2018) Consequences of resistance evolution in a Cas9-based sex conversion-suppression gene drive for insect pest management. *Proceedings of the National Academy of Sciences USA* 115, 6189–6194. doi: 10.1073/pnas.1713825115

Chen, X., Cao, Y., Zhan, S., Zhang, Y., Tan, A. and Huang, Y. (2018) Identification of *yellow* gene family in *Agrotis ipsilon* and functional analysis of *Aiyellow-y* by CRISPR/Cas9. *Insect Biochemistry and Molecular Biology* 94, 1–9. doi: 10.1016/j.ibmb.2018.01.002

Chen, X., Cao, Y., Zhan, S., Tan, A., Palli, S.R. and Huang, Y. (2019) Disruption of sex-specific *doublesex* exons results in male- and female-specific defects in the black cutworm, *Agrotis ipsilon*. *Pest Management Science* 75, 1697–1706. doi: 10.1002/ps.5290

Chevalier, B.S., Kortemme, T., Chadsey, M.S., Baker, D., Monnat, R.J. and Stoddard, B.L. (2002) Design, activity, and structure of a highly specific artificial endonuclease. *Molecular Cell* 10, 895–905. doi: 10.1016/s1097-2765(02)00690-1

Chouteau, M., Llaurens, V., Piron-Prunier, F. and Joron, M. (2017) Polymorphism at a mimicry supergene maintained by opposing frequency-dependent selection pressures. *Proceedings of the National Academy of Sciences USA* 114, 8325–8329. doi: 10.1073/pnas.1702482114

Colleaux, L., d'Auriol, L., Betermier, M., Cottarel, G., Jacquier, A. *et al.* (1986) Universal code equivalent of a yeast mitochondrial intron reading frame is expressed into *E. coli* as a specific double strand endonuclease. *Cell* 44, 521–533. doi: 10.1016/0092-8674(86)90262-x

Concha, C., Wallbank, R.W.R., Hanly, J.J., Fenner, J., Livraghi, L. *et al.* (2019) Interplay between developmental flexibility and determinism in the evolution of mimetic *Heliconius* wing patterns. *Current Biology* 29, 3996–4009.e4. doi: 10.1016/j.cub.2019.10.010

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R. *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823. doi: 10.1126/science.1231143

Dong, Z., Dong, F., Yu, X., Huang, L., Jiang, Y. *et al.* (2018) Excision of nucleopolyhedrovirus form transgenic silkworm using the CRISPR/Cas9 system. *Frontiers in Microbiology* 9, 209. doi: 10.3389/fmicb.2018.00209

Dong, Z., Long, J., Huang, L., Hu, Z., Chen, P. *et al.* (2019) Construction and application of an *HSP70* promoter-inducible genome editing system in transgenic silkworm to induce resistance to *Nosema bombycis*. *Applied Microbiology and Biotechnology* 103, 9583–9592. doi: 10.1007/s00253-019-10135-3

Doudna, J.A. and Charpentier, E. (2014) Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* 346, 1258096. doi: 10.1126/science.1258096

Du, Q., Wen, L., Zheng, S.-C., Bi, H.-L., Huang, Y.-P. *et al.* (2019) Identification and functional characterization of *doublesex* gene in the testis of *Spodoptera litura*. *Insect Science* 26, 1000–1010. doi: 10.1111/1744-7917.12608

Dujon, B. (1989) Group I introns as mobile genetic elements: facts and mechanistic speculations – a review. *Gene* 82, 91–114. doi: 10.1016/0378-1119(89)90034-6

Enciso-Romero, J., Pardo-Díaz, C., Martin, S.H., Arias, C.F., Linares, M. *et al.* (2017) Evolution of novel mimicry rings facilitated by adaptive introgression in tropical butterflies. *Molecular Ecology* 26, 5160–5172. doi: 10.1111/mec.14277

Erickson, P.A., Ellis, N.A. and Miller, C.T. (2016) Microinjection for transgenesis and genome editing in threespine sticklebacks. *JoVE (Journal of Visualized Experiments)* 111, 54055. doi: 10.3791/54055

Farboud, B. and Meyer, B.J. (2015) Dramatic enhancement of genome editing by CRISPR/Cas9 through improved guide RNA design. *Genetics* 199, 959–971. doi: 10.1534/genetics.115.175166

Farnworth, M.S., Eckermann, K.N., Ahmed, H.M.M., Mühlen, D.S., He, B. and Bucher, G. (2020) The red flour beetle as model for comparative neural development: genome editing to mark neural cells in

Tribolium brain development. In: Sprecher, S.G. (ed.) *Brain Development: Methods and Protocols*. Springer, New York, pp. 191–217. doi: 10.1007/978-1-4939-9732-9_11

Foster, S.D., Glover, S.R., Turner, A.N., Chatti, K. and Challa, A.K. (2019) A mixing heteroduplex mobility assay (mHMA) to genotype homozygous mutants with small indels generated by CRISPR-Cas9 nucleases. *MethodsX* 6, 1–5. doi: 10.1016/j.mex.2018.11.017

Fraser, M.J. (2012) Insect transgenesis: current applications and future prospects. *Annual Review of Entomology* 57, 267–289. doi: 10.1146/annurev.ento.54.110807.090545

Gallant, J.R., Imhoff, V.E., Martin, A., Savage, W.K., Chamberlain, N.L. *et al.* (2014) Ancient homology underlies adaptive mimetic diversity across butterflies. *Nature Communications* 5, 4817. doi: 10.1038/ncomms5817

Gantz, V.M. and Bier, E. (2015) Genome editing. The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. *Science* 348, 442–444. doi: 10.1126/science.aaa5945

Gantz, V.M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V.M. *et al.* (2015) Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. *Proceedings of the National Academy of Sciences USA* 112, E6736–E6743. doi: 10.1073/pnas.1521077112

Garneau, J.E., Dupuis, M.-È., Villion, M., Romero, D.A., Barrangou, R. *et al.* (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* 468, 67–71. doi: 10.1038/nature09523

Gasiunas, G., Barrangou, R., Horvath, P. and Siksnys, V. (2012) Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences USA* 109, E2579–E2586. doi: 10.1073/pnas.1208507109

Gloor, G.B., Nassif, N.A., Johnson-Schlitz, D.M., Preston, C.R. and Engels, W.R. (1991) Targeted gene replacement in *Drosophila* via P element-induced gap repair. *Science* 253, 1110–1117. doi: 10.1126/science.1653452

Gratz, S.J., Cummings, A.M., Nguyen, J.N., Hamm, D.C., Donohue, L.K. *et al.* (2013) Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. *Genetics* 194, 1029–1035. doi: 10.1534/genetics.113.152710

Gratz, S.J., Ukken, F.P., Rubinstein, C.D., Thiede, G., Donohue, L.K. *et al.* (2014) Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in *Drosophila*. *Genetics* 196, 961–971. doi: 10.1534/genetics.113.160713

Gratz, S.J., Harrison, M.M., Wildonger, J. and O'Connor-Giles, K.M. (2015) Precise genome editing of *Drosophila* with CRISPR RNA- https://doi.org/10.1007/978-1-4939-2687-9_22

Hall, A.B., Basu, S., Jiang, X., Qi, Y., Timoshevskiy, V.A. *et al.* (2015) SEX DETERMINATION. A male-determining factor in the mosquito *Aedes aegypti*. *Science* 348, 1268–1270. doi: 10.1126/science.aaa2850

Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C. *et al.* (2016) A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae. Nature Biotechnology* 34, 78–83. doi: 0.1038/nbt.3439

Handler, A.M. (2004) Understanding and improving transgene stability and expression in insects for SIT and conditional lethal release programs. *Insect Biochemistry and Molecular Biology* 34, 121–130. doi: 10.1016/j.ibmb.2003.08.005

Handler, A.M. and Atkinson, P.W. (2006) Insect transgenesis: mechanisms, applications, and ecological safety. *Biotechnology and Genetic Engineering Reviews* 23, 129–156. doi: 10.1080/02648725.2006.10648082

Heinze, S.D., Kohlbrenner, T., Ippolito, D., Meccariello, A., Burger, A. *et al.* (2017) CRISPR-Cas9 targeted disruption of the *yellow* ortholog in the housefly identifies the brown body locus. *Scientific Reports* 7, 4582. doi: 10.1038/s41598-017-04686-6

Housden, B.E. and Perrimon, N. (2016) Detection of indel mutations in *Drosophila* by high-resolution melt analysis (HRMA). *Cold Spring Harbor Protocols* 2016(9), 794–801. doi: 10.1101/pdb.prot090795.

Housden, B.E., Lin, S. and Perrimon, N. (2014) Cas9-based genome editing in *Drosophila*. In: Doudna, J.A. and Sontheimer, E.J. (eds) *Methods in Enzymology*, Vol. 546: *The Use of CRISPR/Cas9, ZFNs, and TALENs in Generating Site-Specific Genome Alterations*. Elsevier, San Diego, California, pp. 415–439. doi: 10.1016/B978-0-12-801185-0.00019-2

Hoy, M.A. (2000) Transgenic arthropods for pest management programs: risks and realities. *Experimental & Applied Acarology* 24, 463–495. doi: 10.1023/a:1006401225083

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M. and Nakata, A. (1987) Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *Journal of Bacteriology* 169, 5429–5433. doi: 10.1128/jb.169.12.5429-5433.1987

Ito, K., Katsuma, S., Kuwazaki, S., Jouraku, A., Fujimoto, T. *et al.* (2016) Mapping and recombination analysis of two moth colour mutations, Black moth and Wild wing spot, in the silkworm *Bombyx mori*. *Heredity* 116, 52–59. doi: 10.1038/hdy.2015.69

Jacquier, A. and Dujon, B. (1985) An intron-encoded protein is active in a gene conversion process that spreads an intron into a mitochondrial gene. *Cell* 41, 383–394. doi: 10.1016/s0092-8674(85)80011-8

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821. doi: 10.1126/science.1225829

Joron, M., Papa, R., Beltrán, M., Chamberlain, N., Mavárez, J. *et al.* (2006) A conserved supergene locus controls colour pattern diversity in *Heliconius* butterflies. *PLoS Biology* 4, e303. doi: 10.1371/journal.pbio.0040303

Khan, S.A., Reichelt, M. and Heckel, D.G. (2017) Functional analysis of the ABCs of eye color in *Helicoverpa armigera* with CRISPR/Cas9-induced mutations. *Scientific Reports* 7, 40025. doi: 10.1038/srep40025

Kim, J.M., Kim, D., Kim, S. and Kim, J.-S. (2014) Genotyping with CRISPR-Cas-derived RNA-guided endonucleases. *Nature Communications* 5, 3157. doi: 0.1038/ncomms4157

Kim, Y.G., Cha, J. and Chandrasegaran, S. (1996) Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proceedings of the National Academy of Sciences USA* 93, 1156–1160. doi: 10.1073/pnas.93.3.1156

Kistler, K.E., Vosshall, L.B. and Matthews, B.J. (2015) Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*. *Cell Reports* 11, 51–60. doi: 10.1016/j.celrep.2015.03.009

Koidou, V., Denecke, S., Ioannidis, P., Vlatakis, I., Livadaras, I. and Vontas, J. (2020) Efficient genome editing in the olive fruit fly, *Bactrocera oleae*. *Insect Molecular Biology* 29, 363–372. doi: 10.1111/imb.12640

Kondo, S. and Ueda, R. (2013) Highly improved gene targeting by germline-specific Cas9 expression in *Drosophila*. *Genetics* 195, 715–721. doi: 10.1534/genetics.113.156737

Kyrou, K., Hammond, A.M., Galizi, R., Kranjc, N., Burt, A. *et al.* (2018) A CRISPR-Cas9 gene drive targeting *doublesex* causes complete population suppression in caged *Anopheles gambiae* mosquitoes. *Nature Biotechnology* 36, 1062–1066. doi: 10.1038/nbt.4245

Labuhn, M., Adams, F.F., Ng, M., Knoess, S., Schambach, A. *et al.* (2018) Refined sgRNA efficacy prediction improves large- and small-scale CRISPR–Cas9 applications. *Nucleic Acids Research* 46, 1375–1385. doi: 10.1093/nar/gkx1268

Ledford, H. and Callaway, E. (2020) Pioneers of revolutionary CRISPR gene editing win chemistry Nobel. *Nature* 586, 346–347. doi: 10.1038/d41586-020-02765-9

Lewis, J.J., Geltman, R.C., Pollak, P.C., Rondem, K.E., Van Belleghem, S.M. *et al.* (2019) Parallel evolution of ancient, pleiotropic enhancers underlies butterfly wing pattern mimicry. *Proceedings of the National Academy of Sciences USA* 116, 24174–24183. doi: 10.1073/pnas.1907068116

Li, F. and Scott, M.J. (2016) CRISPR/Cas9-mediated mutagenesis of the white and Sex lethal loci in the invasive pest, *Drosophila suzukii*. *Biochemical and Biophysical Research Communications* 469, 911–916. doi: 10.1016/j.bbrc.2015.12.081

Li, J. and Handler, A.M. (2017) Temperature-dependent sex-reversal by a transformer-2 gene-edited mutation in the spotted wing drosophila, *Drosophila suzukii*. *Scientific Reports* 7, 12363. doi: 10.1038/s41598-017-12405-4

Li, J. and Handler, A.M. (2019) CRISPR/Cas9-mediated gene editing in an exogenous transgene and an endogenous sex determination gene in the Caribbean fruit fly, *Anastrepha suspensa*. *Gene* 691, 160–166. doi: 10.1016/j.gene.2018.12.055

Li, M., Au, L.Y.C., Douglah, D., Chong, A., White, B.J. *et al.* (2017) Generation of heritable germline mutations in the jewel wasp *Nasonia vitripennis* using CRISPR/Cas9. *Scientific Reports* 7, 901. doi: 10.1038/s41598-017-00990-3

Liu, G., Liu, W., Zhao, R., He, J., Dong, Z. *et al.* (2021) Genome-wide identification and gene-editing of pigment transporter genes in the swallowtail butterfly *Papilio xuthus*. *BMC Genomics* 22, 120. doi: 10.1186/s12864-021-07400-z.

Liu, P., Jin, B., Li, X., Zhao, Y., Gu, J. *et al.* (2020a) Nix is a male-determining factor in the Asian tiger mosquito *Aedes albopictus. Insect Biochemistry and Molecular Biology* 118, 103311. doi: 10.1016/j.ibmb.2019.103311

Liu, T., Yang, W.-Q., Xie, Y.-G., Liu, P.-W., Xie, L.-H. *et al.* (2019) Construction of an efficient genomic editing system with CRISPR/Cas9 in the vector mosquito *Aedes albopictus. Insect Science* 26, 1045–1054. doi: 10.1111/1744-7917.12645

Liu, X.-L., Han, W.-K., Ze, L.-J., Peng, Y.-C., Yang, Y.-L. *et al.* (2020b) Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 mediated knockout reveals functions of the *yellow-y* gene in *Spodoptera litura*. *Frontiers in Physiology* 11, 615391. doi: 10.3389/fphys.2020.615391

Livraghi, L., Martin, A., Gibbs, M., Braak, N., Arif, S. and Breuker, C.J. (2018) CRISPR/Cas9 as the key to unlocking the secrets of butterfly wing pattern development and its evolution. In: ffrench-Constant, R.H. (ed.) *Advances in Insect Physiology*, Vol. 54: *Butterfly Wing Patterns and* Mimicry. Academic Press, London, pp. 85–115. doi: 10.1016/bs.aiip.2017.11.001

Livraghi, L., Hanly, J.J., Van Bellghem, S.M., Montejo-Kovacevich, G., van der Heijden, E.S. *et al.* (2021) *Cortex cis-regulatory switches establish scale colour identity and pattern diversity in Heliconius.* eLife 10, e68549. doi: 10.7554/eLife.68549

Mackenzie, S.M., Brooker, M.R., Gill, T.R., Cox, G.B., Howells, A.J. and Ewart, G.D. (1999) Mutations in the *white* gene of *Drosophila melanogaster* affecting ABC transporters that determine eye colouration. *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1419, 173–185. doi: 10.1016/S0005-2736(99)00064-4

Makarova, K.S., Haft, D.H., Barrangou, R., Brouns, S.J.J., Charpentier, E. *et al.* (2011) Evolution and classification of the CRISPR-Cas systems. *Nature Reviews Microbiology* 9, 467–477. doi: 10.1038/nrmicro2577

Malina, A., Cameron, C.J.F., Robert, F., Blanchette, M., Dostie, J. and Pelletier, J. (2015) PAM multiplicity marks genomic target sites as inhibitory to CRISPR-Cas9 editing. *Nature Communications* 6, 10124. doi: 10.1038/ncomms10124

Markert, M.J., Zhang, Y., Enuameh, M.S., Reppert, S.M., Wolfe, S.A. and Merlin, C. (2016) Genomic access to Monarch migration using TALEN and CRISPR/Cas9-mediated targeted mutagenesis. *G3: Genes, Genomes, Genetics* 6, 905–915. doi: 10.1534/g3.116.027029

Martin, A. and Reed, R.D. (2014) *Wnt* signaling underlies evolution and development of the butterfly wing pattern symmetry systems. *Developmental Biology* 395, 367–378. doi: 10.1016/j.ydbio.2014.08.031

Martin, A., Papa, R., Nadeau, N.J., Hill, R.I., Counterman, B.A. *et al.* (2012) Diversification of complex butterfly wing patterns by repeated regulatory evolution of a *Wnt* ligand. *Proceedings of the National Academy of Science USA* 109, 12632–12637. doi: 0.1073/pnas.1204800109.

Martin, A., McCulloch, K.J., Patel, N.H., Briscoe, A.D., Gilbert, L.E. and Reed, R.D. (2014) Multiple recent co-options of *Optix* associated with novel traits in adaptive butterfly wing radiations. *EvoDevo* 5, 7. doi: 10.1186/2041-9139-5-7

Mashal, R.D., Koontz, J. and Sklar, J. (1995) Detection of mutations by cleavage of DNA heteroduplexes with bacteriophage resolvases. *Nature Genetics* 9, 177–183. doi: 10.1038/ng0295-177

Mazo-Vargas, A., Concha, C., Livraghi, L., Massardo, D., Wallbank, R.W.R. *et al.* (2017) Macroevolutionary shifts of *WntA* function potentiate butterfly wing-pattern diversity. *Proceedings of the National Academy of Sciences USA* 114, 10701–10706. doi: 10.1073/pnas.1708149114

McMillan, W.O., Livraghi, L., Concha, C. and Hanly, J.J. (2020) From patterning genes to process: unraveling the gene regulatory networks that pattern *Heliconius* wings. *Frontiers in Ecology and Evolution* 8, 221. doi: 10.3389/fevo.2020.00221

Meccariello, A., Monti, S.M., Romanelli, A., Colonna, R., Primo, P. *et al.* (2017) Highly efficient DNA-free gene disruption in the agricultural pest *Ceratitis capitata* by CRISPR-Cas9 ribonucleoprotein complexes. *Scientific Reports* 7, 10061. doi: 10.1038/s41598-017-10347-5

Meccariello, A., Salvemini, M., Primo, P., Hall, B., Koskinioti, P. *et al.* (2019) *Maleness-on-the-Y* (*MoY*) orchestrates male sex determination in major agricultural fruit fly pests. *Science* 365, 1457–1460. doi: 10.1126/science.aax1318

Meccariello, A., Tsoumani, K.T., Gravina, A., Primo, P., Buonanno, M. *et al.* (2020) Targeted somatic mutagenesis through CRISPR/Cas9 ribonucleoprotein complexes in the olive fruit fly, *Bactrocera oleae*. *Archives of Insect Biochemistry and Physiology* 104, e21667. doi: 10.1002/arch.21667

Merrill, R.M., Dasmahapatra, K.K., Davey, J.W., Dell'Aglio, D.D., Hanly, J.J. *et al.* (2015) The diversification of *Heliconius* butterflies: what have we learned in 150 years? *Journal of Evolutionary Biology* 28, 1417–1438. doi: 10.1111/jeb.12672

Merrill, R.M., Rastas, P., Martin, S.H., Melo, M.C., Barker, S. *et al.* (2019) Genetic dissection of assortative mating behavior. *PLoS Biology* 17, e2005902. doi: 10.1371/journal.pbio.2005902

Miller, D.F.B., Holtzman, S.L. and Kaufman, T.C. (2002) Customized microinjection glass capillary needles for P-element transformations in *Drosophila melanogaster*. *BioTechniques* 33, 366–367, 369–370, 372 passim. doi: 10.2144/02332rr03

Mojica, F.J., Díez-Villaseñor, C., Soria, E. and Juez, G. (2000) Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. *Molecular Microbiology* 36, 244–246. doi: 10.1046/j.1365-2958.2000.01838.x

Mojica, F.J.M., Díez-Villaseñor, C., García-Martínez, J. and Soria, E. (2005) Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *Journal of Molecular Evolution* 60, 174–182. doi: 10.1007/s00239-004-0046-3

Monteiro, A. (2012) Gene regulatory networks reused to build novel traits: co-option of an eye-related gene regulatory network in eye-like organs and red wing patches on insect wings is suggested by *optix* expression. *BioEssays* 34, 181–186. doi: 10.1002/bies.201100160

Moreno-Mateos, M.A., Vejnar, C.E., Beaudoin, J.-D., Fernandez, J.P., Mis, E.K. *et al.* (2015) CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. *Nature Methods* 12, 982–988. doi: 10.1038/nmeth.3543

Nadeau, N.J., Pardo-Diaz, C., Whibley, A., Supple, M.A., Saenko, S.V. *et al.* (2016) The gene *cortex* controls mimicry and crypsis in butterflies and moths. *Nature* 534, 106–110. doi: 10.1038/nature17961

Nie, H.-Y., Liang, L.-Q., Li, Q.-F., Li, Z.-H.-Q., Zhu, Y.-N. *et al.* (2021) CRISPR/Cas9 mediated knockout of *Amyellow-y* gene results in melanization defect of the cuticle in adult *Apis mellifera*. *Journal of Insect Physiology* 132, 104264. doi: 10.1016/j.jinsphys.2021.104264

Organista, M.F., Martín, M., de Celis, J.M., Barrio, R., López-Varea, A. *et al.* (2015) The Spalt transcription factors generate the transcriptional landscape of the *Drosophila melanogaster* wing pouch central region. *PLoS Genetics* 11, e1005370. doi: 10.1371/journal.pgen.1005370

Paula, D.P. (2021) Next-generation sequencing and its impacts on entomological research in ecology and evolution. *Neotropical Entomology* 50, 679–696. doi: 10.1007/s13744-021-00895-x

Paulo, D.F., Williamson, M.E., Arp, A.P., Li, F., Sagel, A. *et al.* (2019) Specific gene disruption in the major livestock pests *Cochliomyia hominivorax* and *Lucilia cuprina* using CRISPR/Cas9. *G3: Genes. Genomes. Genetics* 9, 3045–3055. doi: 10.1534/g3.119.400544

Paulo, D.F., Junqueira, A.C.M., Arp, A.P., Vieira, A.S., Ceballos, J. *et al.* (2021) Disruption of the odorant coreceptor *Orco* impairs foraging and host finding behaviors in the New World screwworm fly. *Scientific Reports* 11, 11379. doi: 10.1038/s41598-021-90649-x

Peng, W., Yu, S., Handler, A.M., Tu, Z., Saccone, G. *et al.* X(2020) miRNA-1-3p is an early embryonic male sex-determining factor in the Oriental fruit fly *Bactrocera dorsalis. Nature Communications* 11, 932. doi: 10.1038/s41467-020-14622-4

Port, F., Chen, H.-M., Lee, T. and Bullock, S.L. (2014) Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in *Drosophila. Proceedings of the National Academy of Sciences USA* 111, E2967–E2976. doi: 10.1073/pnas.1405500111

Port, F., Starostecka, M. and Boutros, M. (2020a) Multiplexed conditional genome editing with Cas12a in *Drosophila. Proceedings of the National Academy of Sciences USA* 117, 22890–22899. doi: 10.1073/pnas.2004655117

Port, F., Strein, C., Stricker, M., Rauscher, B., Heigwer, F. *et al.* (2020b) A large-scale resource for tissue-specific CRISPR mutagenesis in *Drosophila. eLife* 9, e53865. doi: 10.7554/eLife.53865

Primo, P., Meccariello, A., Inghilterra, M.G., Gravina, A., Del Corsano, G. *et al.* (2020) Targeting the autosomal *Ceratitis capitata* transformer gene using Cas9 or dCas9 to masculinize XX individuals without inducing mutations. *BMC Genetics* 21, 150. doi: 10.1186/s12863-020-00941-4

Reed, R.D., Papa, R., Martin, A., Hines, H.M., Counterman, B.A. *et al.* (2011) *optix* drives the repeated convergent evolution of butterfly wing pattern mimicry. *Science* 333, 1137–1141. doi: 10.1126/science.1208227

Ren, X., Sun, J., Housden, B.E., Hu, Y., Roesel, C. *et al.* (2013) Optimized gene editing technology for *Drosophila melanogaster* using germ line-specific Cas9. *Proceedings of the National Academy of Sciences USA* 110, 19012–19017. doi: 10.1073/pnas.1318481110.

Ren, X., Yang, Z., Xu, J., Sun, J., Mao, D. *et al.* (2014) Enhanced specificity and efficiency of the CRISPR/Cas9 system with optimized sgRNA parameters in *Drosophila. Cell Reports* 9, 1151–1162. doi: 10.1016/j.celrep.2014.09.044

Rudin, N., Sugarman, E. and Haber, G.E. (1989) Genetic and physical analysis of double-strand break repair and recombination in *Saccharomyces cerevisiae*. *Genetics* 122(3), 519–534. doi: 10.1093/genetics/122.3.519

Sander, J.D. and Joung, J.K. (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature Biotechnology* 32, 347–355. doi: 10.1038/nbt.2842

Sebo, Z.L., Lee, H.B., Peng, Y. and Guo, Y. (2014) A simplified and efficient germline-specific CRISPR/Cas9 system for *Drosophila* genomic engineering. *Fly (Austin)* 8, 52–57. doi: 10.4161/fly.26828.

Seimiya, M. and Gehring, W.J. (2000) The *Drosophila* homeobox gene *optix* is capable of inducing ectopic eyes by an eyeless-independent mechanism. *Development* 127, 1879–1886.

Sekelsky, J. (2017) DNA repair in *Drosophila*: mutagens, models, and missing genes. *Genetics* 205, 471–490. doi: 10.1534/genetics.116.186759

Shah, S.A., Erdmann, S., Mojica, F.J.M. and Garrett, R.A. (2013) Protospacer recognition motifs: mixed identities and functional diversity. *RNA Biology* 10, 891–899. doi: 10.4161/rna.23764

Sharma, A., Heinze, S.D., Wu, Y., Kohlbrenner, T., Morilla, I. *et al.* (2017) Male sex in houseflies is determined by *Mdmd*, a paralog of the generic splice factor gene *CWC22*. *Science* 356, 642–645. doi: 10.1126/science.aam5498

Sim, S.B., Kauwe, A.N., Ruano, R.E.Y., Rendon, P. and Geib, S.M. (2019) The ABCs of CRISPR in Tephritidae: developing methods for inducing heritable mutations in the genera *Anastrepha, Bactrocera* and *Ceratitis. Insect Molecular Biology* 28, 277–289. doi: 10.1111/imb.12550

Simoni, A., Hammond, A.M., Beaghton, A.K., Galizi, R., Taxiarchi, C. *et al.* (2020) A male-biased sex-distorter gene drive for the human malaria vector *Anopheles gambiae*. *Nature Biotechnology* 38, 1054–1060. doi: 10.1038/s41587-020-0508-1

Sobels, F.H. (1985) Studies in comparative chemical mutagenesis. *Environmental Mutagenesis* 7, 759–773. doi: 10.1002/em.2860070513

Sosnowski, B.A., Belote, J.M. and McKeown, M. (1989) Sex-specific alternative splicing of RNA from the *transformer* gene results from sequence-dependent splice site blockage. *Cell* 58, 449–459. doi: 10.1016/0092-8674(89)90426-1

Stern, D.L. and Orgogozo, V. (2009) Is genetic evolution predictable? *Science* 323, 746–751. doi: 10.1126/science.1158997

Tanaka, R., Murakami, H., Ote, M. and Yamamoto, D. (2016) Clustered regulatory interspaced short palindromic repeats (CRISPR)-mediated mutagenesis and phenotype rescue by *piggyBac* transgenesis in a nonmodel *Drosophila* species. *Insect Molecular Biology* 25, 355–361. doi: 10.1111/imb.12232

Thomas, K.R., Folger, K.R. and Capecchi, M.R. (1986) High frequency targeting of genes to specific sites in the mammalian genome. *Cell* 44, 419–428. doi: 10.1016/0092-8674(86)90463-0

Van Belleghem, S.M., Rastas, P., Papanicolaou, A., Martin, S.H., Arias, C.F. *et al.* (2017) Complex modular architecture around a simple toolkit of wing pattern genes. *Nature Ecology & Evolution* 1, 52. doi: 10.1038/s41559-016-0052

Van Belleghem, S.M., Lewis, J.J., Rivera, E.S. and Papa, R. (2021) *Heliconius* butterflies: a window into the evolution and development of diversity. *Current Opinion in Genetics & Development* 69, 72–81. doi: 10.1016/j.gde.2021.01.010

van der Oost, J., Westra, E.R., Jackson, R.N. and Wiedenheft, B. (2014) Unravelling the structural and mechanistic basis of CRISPR-Cas systems. *Nature Reviews Microbiology* 12, 479–492. doi: 10.1038/nrmicro3279.

VanKuren, N.W., Massardo, D., Nallu, S. and Kronforst, M.R. (2019) Butterfly mimicry polymorphisms highlight phylogenetic limits of gene reuse in the evolution of diverse adaptations. *Molecular Biology and Evolution* 36, 2842–2853. doi: 10.1093/molbev/msz194

Van't Hof, A.E., Campagne, P., Rigden, D.J., Yung, C.J., Lingley, J. *et al.* (2016) The industrial melanism mutation in British peppered moths is a transposable element. *Nature* 534, 102–105. doi: 10.1038/nature17951

Van't Hof, A.E., Reynolds, L.A., Yung, C.J., Cook, L.M. and Saccheri, I.J. (2019) Genetic convergence of industrial melanism in three geometrid moths. *Biology Letters* 15, 20190582. doi: 10.1098/rsbl.2019.0582

Wallbank, R.W.R., Baxter, S.W., Pardo-Diaz, C., Hanly, J.J., Martin, S.H. *et al.* (2016) Evolutionary novelty in a butterfly wing pattern through enhancer shuffling. *PLoS Biology* 14, e1002353. doi: 10.1371/journal.pbio.1002353

Wallrath, L.L. and Elgin, S.C. (1995) Position effect variegation in *Drosophil*a is associated with an altered chromatin structure. *Genes & Development* 9, 1263–1277. doi: 10.1101/gad.9.10.1263

Wang, P., Zhao, Q., Qiu, Z., Bi, S., Wang, W. *et al.* (2019a) The silkworm (*Bombyx mor*i) neuropeptide orcokinin is involved in the regulation of pigmentation. *Insect Biochemistry and Molecular Biology* 114, 103229. doi: 10.1016/j.ibmb.2019.103229.

Wang, Y.-H., Chen, X.-E., Yang, Y., Xu, J., Fang, G.-Q. *et al.* (2019b) The *Masc* gene product controls masculinization in the black cutworm, *Agrotis ipsilon*. *Insect Science* 26, 1037–1044. doi: 0.1111/1744-7917.12635

Wang, Y., Huang, Y., Xu, X., Liu, Z., Li, J. *et al.* (2020) CRISPR/Cas9-based functional analysis of *yellow* gene in the diamondback moth, *Plutella xylostella. Insect Science* 28, 1504–1509. doi: 10.1111/1744-7917.12870

Wang, Z., Gerstein, M. and Snyder, M. (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics* 10, 57–63. doi: 10.1038/nrg2484

Ward, C.M., Aumann, R.A., Whitehead, M.A., Nikolouli, K., Leveque, G. *et al.* (2021) *White pupae* phenotype of tephritids is caused by parallel mutations of a MFS transporter. *Nature Communications* 12, 491. doi: 10.1038/s41467-020-20680-5

Werren, J.H., Nur, U. and Wu, C.I. (1988) Selfish genetic elements. *Trends in Ecology & Evolution* 3, 297–302. doi: 10.1016/0169-5347(88)90105-X.

Westerman, E.L., VanKuren, N.W., Massardo, D., Tenger-Trolander, A., Zhang, W. *et al.* (2018) *Aristaless* controls butterfly wing color variation used in mimicry and mate choice. *Current Biology* 28, 3469–3474.e4. doi: 10.1016/j.cub.2018.08.051

Wyman, C. and Kanaar, R. (2006) DNA double-strand break repair: all's well that ends well. *Annual Review of Genetics* 40, 363–383. doi: 10.1146/annurev.genet.40.110405.090451

Xiao, C., Qiu, S. and Robertson, R.M. (2017) The *white* gene controls copulation success in *Drosophila melanogaster*. *Scientific Reports* 7, 7712. doi: 0.1038/s41598-017-08155-y

Xu, X., Harvey-Samuel, T., Yang, J., Alphey, L. and You, M. (2020) Ommochrome pathway genes *kynurenine 3-hydroxylase* and *cardinal* participate in eye pigmentation in *Plutella xylostella*. *BMC Molecular and Cell Biology* 21, 63. doi: 10.1186/s12860-020-00308-8

Xue, Z., Wu, M., Wen, K., Ren, M., Long, L. *et al.* (2014) CRISePR/Cas9 mediates efficient conditional mutagenesis in *Drosophila. G3 (Genes. Genomes. Genetics)* 4, 2167–2173. doi: 10.1534/g3.114.014159

Yan, Y., Ziemek, J. and Schetelig, M.F. (2020) CRISPR/Cas9 mediated disruption of the *white* gene leads to pigmentation deficiency and copulation failure in *Drosophila suzukii. Journal of Insect Physiology* 126, 104091. doi: 10.1016/j.jinsphys.2020.104091

Yan, Y., Kobayashi, Y., Huang, C., Liu, B., Qian, W. *et al.* (2021) Highly efficient temperature inducible CRISPR-Cas9 gene targeting in *Drosophila suzukii. International Journal of Molecular Sciences* 22, 6724. doi: 10.3390/ijms22136724

Yennmalli, R.M., Kalra, S., Srivastava, P.A. and Garlapati, V.K. (2017) Computational tools and resources for CRISPR/Cas 9 genome editing method. *MOJ Proteomics & Bioinformatics* 5, 116–120. doi: 10.15406/mojpb.2017.05.00164

Yu, Z., Ren, M., Wang, Z., Zhang, B., Rong, Y.S. *et al.* (2013) Highly efficient genome modifications mediated by CRISPR/Cas9 in *Drosophila*. *Genetics* 195, 289–291. doi: 10.1534/genetics.113.153825

Yu, Z., Chen, H., Liu, J., Zhang, H., Yan, Y. *et al.* (2014) Various applications of TALEN- and CRISPR/Cas9-mediated homologous recombination to modify the *Drosophila* genome. *Biology Open* 3, 271–280. doi: 0.1242/bio.20147682.

Zhang, L. and Reed, R.D. (2017) A practical guide to CRISPR/Cas9 genome editing in Lepidoptera. In: Sekimura, T. and Nijhout, H.F. (eds) *Diversity and Evolution of Butterfly Wing Patterns: An Integrative Approach.* Springer, Singapore, pp. 155–172. doi: 10.1007/978-981-10-4956-9_8

Zhang, L., Mazo-Vargas, A. and Reed, R.D. (2017) Single master regulatory gene coordinates the evolution and development of butterfly color and iridescence. *Proceedings of the National Academy of Sciences USA* 114, 10707–10712. doi: 10.1073/pnas.1709058114

Zhang, R., Zhang, Z., Huang, Y., Qian, A. and Tan, A. (2020) A single ortholog of *teashirt* and *tiptop* regulates larval pigmentation and adult appendage patterning in *Bombyx mori*. *Insect Biochemistry and Molecular Biology* 121, 103369. doi: 10.1016/j.ibmb.2020.103369

Zhang, X., Xia, L., Day, B.A., Harris, T.I., Oliveira, P. *et al.* (2019) CRISPR/Cas9 initiated transgenic silkworms as a natural spinner of spider silk. *Biomacromolecules* 20, 2252–2264. doi: 10.1021/acs.biomac.9b00193

Zhao, S., Xing, Z., Liu, Z., Liu, Y., Liu, X. *et al.* (2019) Efficient somatic and germline genome engineering of *Bactrocera dorsalis* by the CRISPR/Cas9 system. *Pest Management Science* 75, 1921–1932. doi: 10.1002/ps.5305

Zhu, G.-H., Peng, Y.-C., Zheng, M.-Y., Zhang, X.-Q., Sun, J.-B. *et al.* (2017) CRISPR/Cas9 mediated *BLOS2* knockout resulting in disappearance of yellow strips and white spots on the larval integument in *Spodoptera litura. Journal of Insect Physiology* 103, 29–35. doi: 10.1016/j.jinsphys.2017.09.008

8 An Introduction to the Molecular Genetics of Gene Drives and Thoughts on Their Gradual Transition to Field Use

Robyn R. Raban and Omar S. Akbari*

Division of Biological Sciences, Section of Cell and Developmental Biology, University of California, San Diego, La Jolla, CA 92093, USA

8.1 Introduction

Pest control has fundamentally impacted the course of humanity. Ever since the first humans began settling into farming communities, pest species have threatened human food security. Pest control methods, including highly toxic chemical-based methods, were being employed by some of the most advanced societies throughout Asia, the Middle East and Europe even thousands of years ago. As populations expanded, the need for secure and reliable food supplies also grew and by the 18th and 19th centuries chemical pest control was widespread. Pest control methods were also being employed against other nuisance pests such as fleas, lice and mosquitoes well before they were known to vector diseases. Once these insects were implicated in the transmission of human diseases, intense efforts to control and eradicate these insects surged. Due to the expansion of available pesticides over the past 80 years, chemical-based pest control methods are commonplace today and their use has been essential to achieving our current levels of food production and reductions in vector-borne disease, but their broad application has been problematic. Effects of pesticides on the environment

have been numerous and well documented and now their omnipresent use has led to widespread development of pesticide resistance. A famous quote from Rachel Carson's book, *Silent Spring*, exemplifies these detrimental effects on the environment:

> If, having endured much, we have at last asserted our 'right to know,' and if by knowing, we have concluded that we are being asked to take senseless and frightening risks, then we should no longer accept the counsel of those who tell us that we must fill our world with poisonous chemicals; we should look about and see what other course is open to us.

In line with Carson's comment to 'see what other course is open to us', we need to reduce our reliance on harmful chemicals by creating a new generation of pest control technologies to support agricultural industries and public health. New genetic-based technologies for the control of disease vectors and agricultural pests have emerged over the past few years, which may be able to support more sustainable pest control. Unlike chemical pesticides, which also kill non-target species and have been shown to accumulate in food chains, these genetic technologies are designed to impact only the target pest species. In this chapter, we introduce the

*Corresponding author, email: oakbari@ucsd.edu

molecular biology of genetic control technologies and compare and contrast how these technologies have been designed to date. We also discuss the benefits, risks and limitations of these technologies as they transition from laboratory studies to field evaluations. While these technologies are unlikely to fully address the centuries-long pest problem, in the future they may have a profound impact on pest management.

Two predominant genetic approaches have been described throughout literature for over a century to control pest organisms (Fig. 8.1). One, known as population modification, aims to impart a favourable trait, such as pathogen resistance, or insecticide susceptibility, into a population. The other approach, known as population suppression, aims to reduce, and possibly eliminate, disease-transmitting populations. In population modification, many synthetic and naturally derived components, known as effectors, have been generated to encode antiviral properties (Franz *et al.*, 2006, 2014; Mathur *et al.*, 2010; Carter *et al.*, 2015; Yen *et al.*, 2018; Buchman *et al.*, 2019a,b) or anti-*Plasmodium* properties (Gwadz *et al.*, 1989; Barreau *et al.*, 1995; Rodriguez *et al.*, 1995; Luckhart *et al.*, 1998; Shahabuddin *et al.*, 1998; Yoshida *et al.*, 1999, 2001, 2007; Conde *et al.*, 2000; de Lara Capurro *et al.*, 2000; Tsai *et al.*, 2001; Vizioli *et al.*, 2001; Zieler *et al.*, 2001; Ito *et al.*,
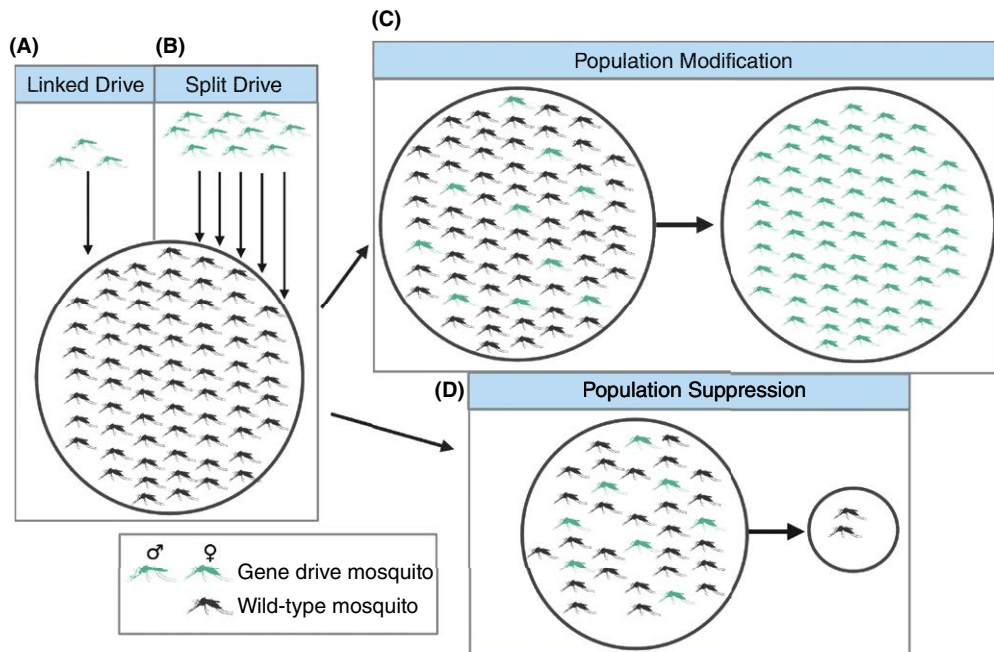
Fig. 8.1. Main categories and goals of population control systems. Gene drive technologies can be designed as **(A)** an autonomous homing linked drive or as **(B)** a non-autonomous homing split drive. If a homing linked drive **(A)** is released into a wild population, it requires minimal releases of a few individuals to achieve fixation of the gene drive in the target population. This linked gene drive design has the capability to spread widely and establish in neighbouring populations. **(B)** A homing split drive requires multiple releases of individuals to achieve fixation of the gene drive in the population, but the spread is more controllable and the gene drive will not establish in neighbouring populations. Both homing linked drives and homing split drives can be designed to modify populations with beneficial characteristics such as disease refractoriness **(C)** or they can be designed to suppress populations **(D)**. For population modification strategies (C), the number of gene drive individuals increases over time until the gene drive reaches fixation or a high frequency in the population. On the other hand, in population suppression (D), as the gene drive reaches a high frequency the population decreases and approaches extinction. Created with BioRender.com

2002; Moreira *et al.*, 2002, 2007; Arrighi *et al.*, 2008; Carballar-Lejarazú *et al.*, 2008; Gao *et al.*, 2009, 2010; Corby-Harris *et al.*, 2010; Dong *et al.*, 2011; Fang *et al.*, 2011; Isaacs *et al.*, 2011; Zhang *et al.*, 2015) (see Bottino-Rojas and James, Chapter 11, this volume) to render mosquitoes refractory to human pathogen transmission. Importantly, when an anti-pathogen effector is introduced into a population and reaches sufficient frequency, the population is predicted to become resistant to the pathogen. Genetic population suppression approaches, on the contrary, strive to impart a fitness load, reduce the reproductive potential, or induce a sex bias on the population, which can lead to its ultimate decline. The underlying molecular genetic mechanisms that are utilized for both population modification and suppression approaches, however, can result in fitness disadvantages to the organism that make it difficult to maintain and spread these modifications into populations. Therefore, in the absence of a mechanism to confer a fitness advantage to the modification, or limitlessly scale the technology, many potentially transformative genetic technologies may consequently not be feasible for effective population control.

Over half a century ago, gene drive technologies were posited as a tool to enhance the capabilities and impact of early transgenic technologies (Sandler and Novitski, 1957; Hamilton, 1967; Curtis, 1968; Serebrovsky *et al.*, 1969). They can address fitness deficiencies associated with genetic technologies by biasing the inheritance of desired genetic modifications, thereby forcing their spread into a population. Without these tools, the release frequencies required to sustain the desired alterations to the population will in many cases simply exceed current technical capabilities and resources, particularly in resource-limited settings. Consequently, gene drives may prove instrumental to making more genetic-based technologies practical for the control of human and veterinary diseases, agricultural pests and invasive species.

While a synthetic gene drive system has yet to be evaluated in the field, many laboratory-based examples of gene drives in both model and target organisms exemplify the diversity in gene drive design, capabilities and performance. Early gene drive designs focused on translocation-based gene drives (Curtis, 1968; Serebrovsky *et al.*, 1969), an underdominance-based system utilizing chromosomal rearrangements, or inversions, unique to the target population (Curtis *et al.*, 1972; Lorimer *et al.*, 1972; Robinson, 1976; Asman *et al.*, 1981). When released in sufficient numbers, the selective advantage of translocation homozygous individuals can maintain and spread the drive into the population, due to heterozygote death caused by the inheritance of unbalanced chromosomes. These early attempts were of limited success, but as technical capabilities improved, innovative gene drive systems were synthetically engineered that achieved biased inheritance in laboratory populations.

Examples of synthetically engineered gene drive systems include underdominance systems, such as engineered reciprocal translocations (see Champer, Chapter 9, this volume) (Buchman *et al.*, 2018b), killer–rescue-based systems (Webster *et al.*, 2020), and even engineering of synthetic species (Buchman *et al.*, 2020; Maselko *et al.*, 2020). Some gene drive designs have taken advantage of the RNA interference (RNAi) technology to generate maternal toxin and zygotic antidote systems, maternal-effect dominant embryonic arrest (*Medea*) (Chen *et al.*, 2007a; Akbari *et al.*, 2014; Buchman *et al.*, 2018a) and synthetic maternal-effect lethal underdominance (UD^MEL)(Akbari *et al.*, 2013). More recent toxin antidote systems, such as cleave-and-rescue (CleaveR), use CRISPR-associated protein 9 (Cas9) and guide RNA (gRNA) to disrupt an essential gene (Oberhofer *et al.*, 2019). In CleaveR, an addictive, recoded, cleavage-resistant copy of the target gene maintains the function of the drive in *trans* and is the basis of the biased inheritance. Endonucleases are also being explored to generate sex-linked drives, which can reduce populations by biasing sex population ratios. One sex-linked system, X-shredder, for example, strives to bias the population sex ratio towards males by integrating the drive into the Y-chromosome and designing the drive to target X-linked sequences during spermatogenesis (Papathanos, *et al.*, 2014; Galizi *et al.*, 2016; see Arien *et al.*, Chapter 10,
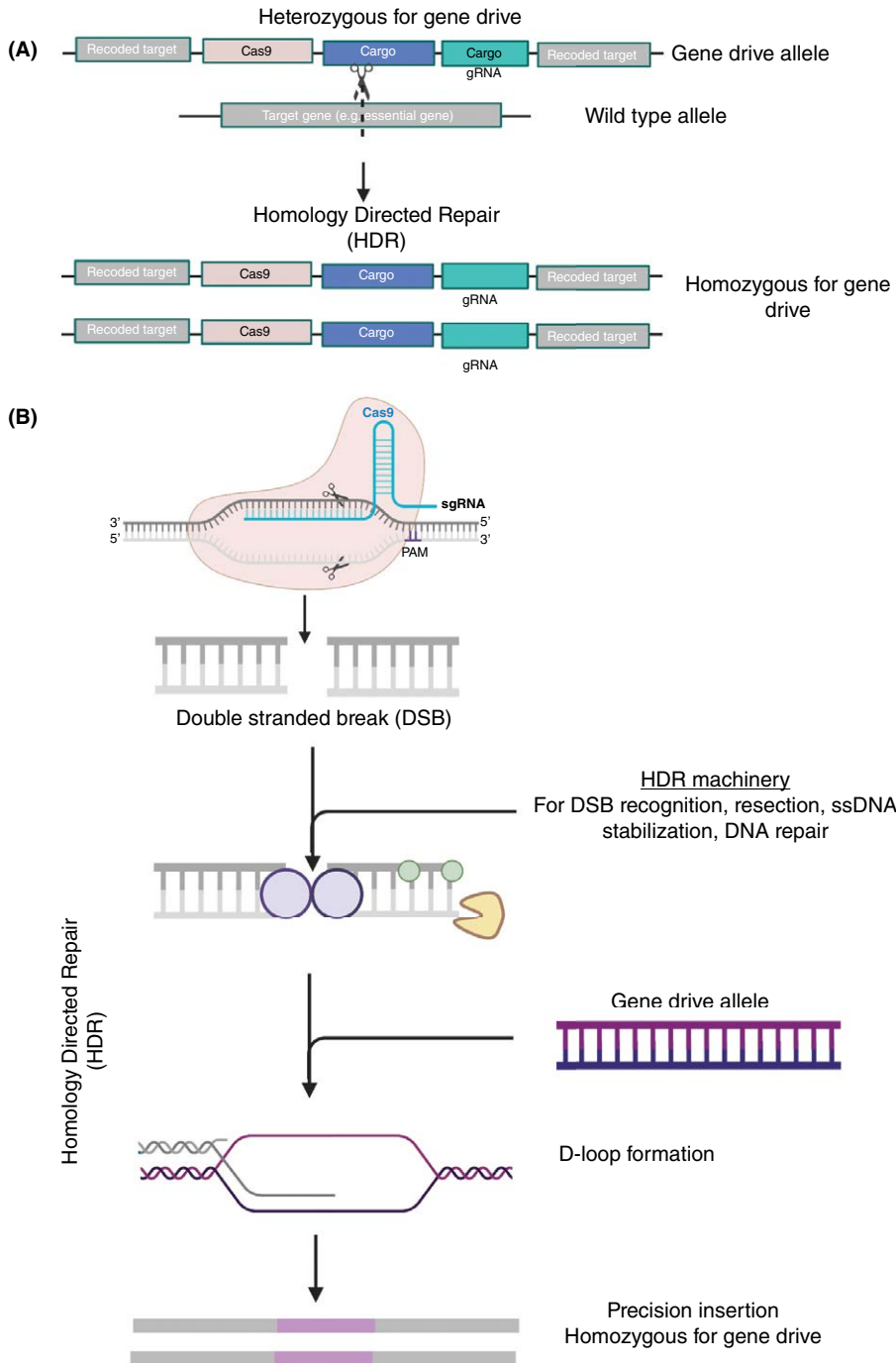
Fig. 8.2. Mechanisms of a homing-based drive system. (A) General schematic of the homology-directed repair (HDR)-mediated copying of a drive allele into a wild-type allele via a CRISPR/Cas9-based gene drive. **(B)** Detailed mechanism of HDR-mediated integration of gene drive allele into wild-type allele. The Cas9 endonuclease cleaves a target site to create a double-strand break (DSB) in the genome.

this volume). More detailed discussions of these gene drives can be found in numerous reviews (Champer *et al.*, 2016; Marshall and Akbari, 2018; Raban *et al.*, 2020), but the overarching focus of this chapter is on one of the more recent and promising gene drive approaches: RNA-guided CRISPR/Cas9-mediated homing endonuclease-based gene drives, referred to as homing gene drives herein, which were first comprehensively articulated by Esvelt *et al.* (2014), following previous homing endonuclease-based design architectures seminally outlined over a decade prior by Burt (2003).

8.2 Molecular Mechanism of CRISPR Homing-based Drive Systems

The advent of Cas9-mediated genome engineering technologies (Jinek *et al.*, 2012; Cong *et al.*, 2013; Mali *et al.*, 2013) has facilitated the precise, programmable genome editing of a wide variety of organisms (see Concha and Papa, Chapter 7, this volume). This versatility stems from the ability to facilely design gRNAs to direct desired site-specific genome cleavage by the Cas9 endonuclease. This cleavage is highly efficient and, when adequately designed, very high cleavage rates can be consistently achieved in multiple organisms. Following cleavage, the ensuing double-strand break (DSB) is repaired by one of the predominant DSB end-joining repair mechanisms, including homology-directed repair (HDR), microhomology-mediated end joining (MMEJ), or non-homologous end joining (NHEJ). The HDR mechanism uses the homologous chromosome copy, sister chromatid, or exogenous DNA as a template to repair the DSB. If the homologous chromosome contains the gene drive allele, then the HDR pathway can facilitate gene conversion of a wild-type allele to a drive allele. When this HDR-based copying occurs in the germline, it provides the basis of biased inheritance for homing-based gene drive technologies into subsequent generations (Fig. 8.2A). The HDR mechanism, however, only occurs during the late S and G2 phases of meiosis when the homologous chromosome is accessible (Branzei and Foiani, 2008). Therefore, the optimal timing of Cas9 activity is an important consideration for directing HDR of the DSB. After generation of the DSB, numerous proteins then recognize, resect and stabilize the ssDNA prior to the displacement and pairing of a homologous DNA strand using a strand from the DNA repair site, creating a displacement loop (D-loop) (Fig. 8.2B). Resolution of the recombination intermediates then completes the repair and when this occurs in the germline it converts offspring heterozygous for the drive to homozygous for the drive. If these drives are linked to a transgene to facilitate a desired gene modification, this mechanism can rapidly spread these modifications throughout a population.

RNA-guided homing-based gene drives developed to date minimally consist of the Cas9 endonuclease expressed in the germline and one or more gRNAs expressed from a ubiquitous polymerase III (pol III) promoter (Esvelt *et al.*, 2014; Champer *et al.*, 2016). How these elements are configured and what genes they are designed to target greatly affect the behaviour and efficiency of the drive (see Champer, Chapter 9, this volume)

Fig. 8.2. Continued.
The actors in the subsequent repair processes vary by taxon, but generally multiple proteins are involved in DSB recognition, resectioning and stabilization (Heyer *et al.*, 2010; Symington and Gautier, 2011; Kowalczykowski, 2015; Symington, 2016; Yang *et al.*, 2020). The resectioning at the 5′ end creates a 3′ overhang (Renkawitz *et al.*, 2014; Bhat and Cortez, 2018), which is stabilized by one or more proteins and is used as a protein substrate and primer for DNA repair (Yang *et al.*, 2020). Then through the assistance of additional protein mediators/modulators a DNA repair protein, commonly RAD51 homologue 1 (RAD51) in many eukaryotes, mediates the identification and strand invasion of the homologous DNA template, resulting in the formation of a displacement loop (D-loop) (San Filippo *et al.*, 2008; Yang *et al.*, 2020). The recombination intermediates are then resolved by one or more pathways based on whether they form single or double Holliday junctions (West, 2009; Heyer *et al.*, 2010; Kowalczykowski, 2015). The end repair results in the copying of the gene drive from one chromosome to the other, which converts an individual that would be heterozygous for the drive to homozygous. Created with BioRender.com

Table 8.1. Key selected papers using Cas9-mediated gene drives.

Purpose	Type	Species	Transmission efficiency in early generations	Cas9 promoter(s)	gRNA target(s)	gRNA promoter	Susceptibility to resistance allele generation	Notable design considerations/highlights	Citation
Initial proof of concept of an RNA-guided homing-based gene drive in flies	Linked	*Drosophila melanogaster*	≥ 95%	*vasa*	*yellow*	U6:3	High	Initial proof of concept in flies; lacked a marker gene to enable drive tracking.	Gantz and Bier, 2015
Study resistance allele formation	Linked	*Drosophila melanogaster*	52–62%	*nanos, vasa*	*yellow*	U6:3	High	Targets X-linked genes, evaluates multiple target sites and Cas9 promoters.	Champer *et al.*, 2017
Study designs to mitigate resistance allele formation	Linked	*Drosophila melanogaster*	38–76%	*nanos, vasa*	*cinnabar, white*	U6:1, U6:3	Lower	Targets X-linked and autosomal genes, multiplex gene targeting and evaluates different Cas9 and gRNA promoters.	Champer *et al.*, 2018
Population suppression	Linked	*Drosophila melanogaster*	≥ 56%	*DNApol-α180, Rcd-1 related, Sry-α*	*transformer*	U6:3	High	Targeting a female essential gene for population control and evaluated promoters with different expression timing.	KaramiNejad Ranjbar *et al.*, 2018
Population suppression	Linked	*Drosophila melanogaster*	0–83%	*nanos*	*deformed, yellow-g*	U6:1, U6:3	High	Evaluated multiplexed gRNAs to reduce resistance allele formation, targeted essential genes.	Oberhofer *et al.*, 2018
Population modification	Linked	*Drosophila melanogaster*	N/A	*nanos*	*melanogaster technical knockout (tko)*	U6:3	Low	Cleave-and-rescue (ClvR) system is homing independent and relies on the high cleavage efficiency of Cas9; targets an essential gene and encodes a cleavage-resistant copy of the target gene (rescue).	Oberhofer *et al.*, 2019

Continued

Table 8.1. Continued.

Purpose	Type	Species	Transmission efficiency in early generations	Cas9 promoter(s)	gRNA target(s)	gRNA promoter	Susceptibility to resistance allele generation	Notable design considerations/highlights	Citation
Population modification	Split	*Drosophila melanogaster*	32–74%	*nanos*	*EGFP (synthetic), yellow*	U6:3	High	Compared linked gene drives, synthetic target site gene drives, and split drive systems; demonstrates that maternal Cas9 protein deposition induces homing in germ cells.	Champer *et al*., 2019b
Study resistance allele formation	Linked	*Drosophila melanogaster*	28–72%	*nanos*	*yellow*	U6:3	High	Evaluated resistance allele formation in genetically diverse strains.	Champer *et al*., 2019a
Population modification	Split	*Drosophila melanogaster*	≥ 69%	*Bicaudal C, nanos, Ubiquitin 63E, vasa*	*white, yellow*	U6:3	High	Included a gRNA–mediated effector to target host genes- relies on the NHEJ–mediated indel formation in somatic tissues; evaluated promoters with different expression timing.	Kandul *et al*., 2020
Population modification	Split	*Drosophila melanogaster*	N/A	*nanos*	*hairy*	U6:3	Low	Toxin-antidote recessive embryo (TARE) drive design; targets a haplosufficient recessive lethal gene (toxin) and encoded cleavage-resistant copy of the gene (antidote); homing independent.	Champer *et al*., 2021b

Population modification	Linked	*Drosophila melanogaster*	N/A	*nanos*	*melanogaster technical knockout, dribble, Transcription-factor-IIA-S*	U6:3	Low	Multiple cleave-and-rescue (ClvR) system elements at the same loci target different haplosufficient essential genes. Each element encodes the rescue from another element to bias inheritance. ClvR is homing independent.	Oberhofer *et al.*, 2020
Population modification	Split	*Drosophila melanogaster*	N/A	*nanos*	*RpL35A*	U6:3	Low	Toxin-antidote recessive embryo (TARE) drive design; targets a haplosufficient recessive lethal gene (toxin) and encoded cleavage resistant copy of the gene (antidote); homing independent.	Champer *et al.*, 2020a
Population modification	Split	*Drosophila melanogaster*	51.5–~100%	*Actin 5C, Beta tubulin 85D, exuperantia, nanos, Rcd-1 related, vasa, ubiquitin 63E*	*DNA polymerase gamma subunit 2*	U6:3	Lower	Home-and-rescue (HomeR) system targets the 3' coding sequence of a haplosufficient essential gene and encodes a linked, dominant rescue of the target gene. Includes an exogenous 3' UTR to prevent recombination events between the drive and the target gene; evaluated multiple Cas9 expression promoters.	Kandul *et al.*, 2021
Population modification	Split	*Drosophila melanogaster*	~64– ~100%	*vasa, nanos*	*prosalpha2, rab5, rab11, spo11*	U6:3	Lower	Targets conserved regions of halplosufficient recessive lethal essential or fertility genes and encodes a linked, dominant rescue of the target gene; evaluated multiple target genes.	Terradas *et al.*, 2021

Continued

Table 8.1. Continued.

Purpose	Type	Species	Transmission efficiency in early generations	Cas9 promoter(s)	gRNA target(s)	gRNA promoter	Susceptibility to resistance allele generation	Notable design considerations/highlights	Citation
Population suppression	Split	*Drosophila melanogaster*	> 86%	*nanos*	*yellow-g*	U6:3	Lower	Multiplexed gRNAs targeting a female essential fertility gene to reduce accumulation of resistance alleles. Fitness costs prevent suppression of laboratory populations.	Yang *et al.*, 2022
Populations modification and suppression	Linked	*Drosophila melanogaster*	52–77%	*nanos*	*hairy* (TARE), *RpL35A* (modification), *yellow-g* (suppression)	U6:3	Lower	Tethered gene drive system using a toxin-antidote recessive embryo (TARE) drive to support the confined establishment of population modification (targeting haplolethal gene) and suppression (targeting female essential haplosufficient gene) drives with multiplexed gRNAs and a linked recoded target gene; homing independent.	Metzloff *et al.*, 2022
Population suppression	Linked	*Drosophila melanogaster*	N/A	*nanos*	*dribble*	U6:3	Low	ClvR system with temperature sensitive intein (self-excising protein) encoded in the rescue transgene, which restores function of the target gene; homing independent.	Oberhofer *et al.*, 2021b
Population modification	Split	*Drosophila melanogaster*	N/A	*nanos*	*melanogaster technical knockout*	U6:3	Low	Evaluated multiple split drive designs of the ClvR system; homing independent.	Oberhofer, *et al.*, 2021a

Population modification	Linked	Anopheles stephensi	≥ 99.5%	vasa	kynurenine hydroxylase	U6A	High	Male germline; homing independent specific transmission, inclusion of blood meal inducible anti-*Plasmodium* effectors.	Gantz *et al.*, 2015
Population suppression	Linked	Anopheles gambiae	91.4–99.6%	vasa2	yellow-g (AGAP005958), nudel (AGAP007280), AGAP011377	U6	High	First demonstration of Cas9-based population suppression in a mosquito vector, targeting a female fertility gene for population control.	Hammond *et al.*, 2016
Population suppression	Linked	Anopheles gambiae	> 95%	zero population growth	doublesex	U6	Lower	Targets a conserved region of a female essential gene to limit resistance allele accumulation.	Kyrou *et al.*, 2018
Population modification	Split	Aedes aegypti	50–94%	4-nitrophenyl phosphatase, exuperentia, nucleoporin 50, trunk, ubiquitin L40	white	U6a,b,c,d	High	First demonstration of a gene drive in *Aedes aegypti* and the first demonstration of a split gene drive in mosquitoes; evaluated multiple gRNA and Cas9 promoters.	Li *et al.*, 2020
Population modification	Split	Aedes aegypti	50–70%	nucleoporin 50, suppressor of defective silencing 3, benign gonial cell neoplasm protein	white	U6b	High	Evaluated the mechanism of biased inheritance in gene drive systems; evaluated multiple Cas9 promoters.	Verkuijl *et al.*, 2020
Population modification	Linked	Anopheles stephensi	57– ~100%	vasa	kynurenine hydroxylase	U6A	Lower	Designed to insert in the *kh* gene where loss of function mutations result in a phenotypic eye change and a reduction in female survival, fertility, and fecundity. Drive includes a recoded copy of the target gene of the *kh* gene.	Adolfi *et al.*, 2020

Continued

Table 8.1. Continued.

Purpose	Type	Species	Transmission efficiency in early generations	Cas9 promoter(s)	gRNA target(s)	gRNA promoter	Susceptibility to resistance allele generation	Notable design considerations/highlights	Citation
Population modification	Linked	*Anopheles gambiae*	> 79%	*vasa, zero population growth*	haplosufficient genes: AGAP011377, AGAP007280, AGAP005958	U6	Lower	Autosomal male-biased sex distorter coupled with a drive.	Simoni *et al.*, 2020
Population modification	Linked	*Anopheles gambiae*	≥ 85.0%	*nanos*	*cardinal/heme peroxidase 6 gene*	U6	High	Evaluated off target drive associated cleavage; more extensive analysis of drive resistant polymorphisms in African populations.	Carballar-Lejarazú *et al.*, 2020
Population modification	Split	*Anopheles gambiae*	> 87%	*vasa*	*peritrophin 1 (AGAP006795), zinc carboxypeptidase A1 (AGAP009593), alkaline phosphatase 2 (AGAP006400)*	U6	Moderate	Integral gene drive design whereby gRNA and effector expression is activated by endogenous genes (hijacks host gene machinery for expression but maintains host gene function); minimized genetic modifications by removing fluorescent markers; incorporated an anti-plasmodium effector.	Hoermann *et al.*, 2021

(Champer *et al.*, 2016) (Table 8.1). When a gene drive is designed to integrate both the Cas9 and gRNA elements at the same genomic loci in a homing linked drive design, these two elements will be inherited together, facilitating rapid spread into a population and likely into other populations (Noble *et al.*, 2018). Therefore, only limited releases of a homing linked drive may be needed to establish the drive in the desired population and beyond.

Another design, referred to as a homing split drive, integrates the Cas9 endonuclease and the gRNA into two or more separate loci (Esvelt *et al.*, 2014; Champer *et al.*, 2016). When a homing split drive is released into a target population, there will be limited co-occurrence of the two independent components and consequently the spread of gene drive is intrinsically limited. In fact, recent modelling has demonstrated that multiple releases of a homing split-drive in *Ae. aegypti* can cause the complete modification of a target population that persists for several years, but spread into neighbouring populations can be deliberately prevented as the co-occurrence of these elements is too low in the neighbouring populations for the drive to establish (Li *et al.*, 2020). Homing split drives, however, require large numbers of individuals to be released and they need to be released multiple times before they reach high enough threshold frequencies to establish and spread within the target population. While homing split-drives offer a more controllable way to safely confine the spread of a gene drive, these additional releases may increase the costs and infrastructure needed to successfully employ a gene drive technology as compared with a homing linked drive.
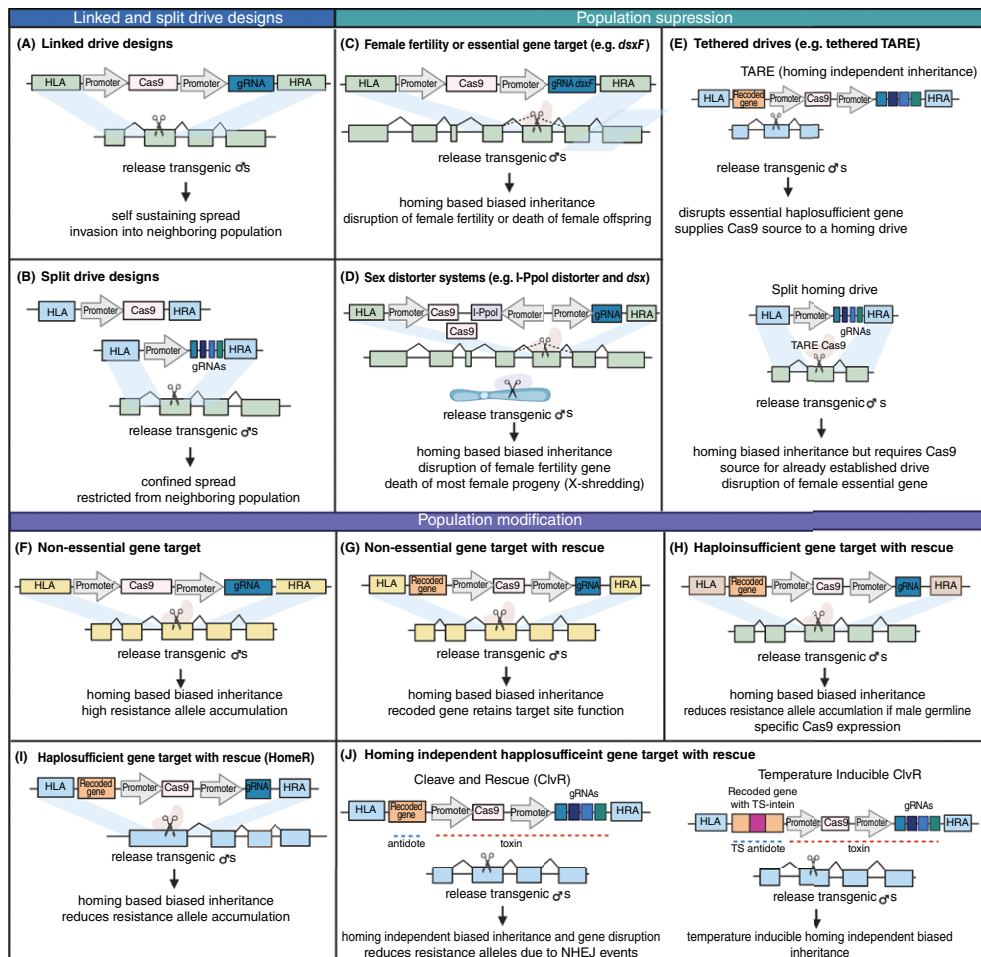
8.3 Population Modification

Population modification strategies retain but alter the target population (see Fig. 8.1C). The benefits of this approach are numerous, but a predominating advantage is its suitability for the prevention of disease resurgence in areas that already achieve substantial reductions in disease transmission. In most areas, some immigration of vectors from neighbouring populations is inevitable and if immigration occurs in an area where the vector has been eliminated, or greatly reduced in numbers, the immigrants, which are capable of transmitting disease, will likely reoccupy this empty niche. Population suppression interventions (see Fig. 8.1D) would need to be continuously implemented in the transition zones to maintain disease-free areas (Champer *et al.*, 2021). Alternatively, when immigrants enter a disease-refractory population, they will breed with the refractory population and their descendants are quickly converted to the disease-refractory phenotype. Population modification is therefore possibly more sustainable and cost effective in the later stages of disease elimination than continuously maintaining suppression technologies in areas that have already achieved substantial disease reduction.

Following the Esvelt *et al.* (2014) CRISPR RNA-guided gene drive blueprints, homing gene drives for population modification were quickly engineered by many groups for a variety of organisms, including model organisms such *Drosophila melanogaster* (Gantz and Bier, 2015; Champer *et al.*, 2018; Carrami *et al.*, 2018; Oberhofer *et al.*, 2018; Champer *et al.*, 2020a; Kandul *et al.*, 2020, 2021; Terradas *et al.*, 2021) (see Champer, Chapter 9, this volume) as well as important pest species such as malaria vectors *Anopheles stephensi* (Gantz *et al.*, 2015; Adolfi *et al.*, 2020) and *Anopheles gambiae* (Hammond *et al.*, 2016; Carballar-Lejarazú *et al.*, 2020) and even the major yellow fever and dengue vector *Aedes aegypti* (Li *et al.*, 2020; Verkuijl *et al.*, 2020). These systems (Fig. 8.3) are the most versatile drive systems to date and are closest in terms of technical development for field trials. Work in anopheline malaria vectors, for example, has advanced from early proof-of-concept work (Gantz *et al.*, 2015; Hammond *et al.*, 2016) and has now demonstrated many improvements in efficiency that may provide feasibility for field trials. Recent drive systems for population modification of *An. gambiae*, a prominent malaria vector found throughout sub-Saharan Africa, achieved > 95% drive efficiency with minimal fitness costs (Carballar-Lejarazú *et al.*, 2020). This homing linked drive was designed with a germline-specific Cas9 linked

Fig. 8.3. Examples of key population modification and suppression gene drive designs. (A, B) General design of **(A)** linked and **(B)** split drive systems. **(C–E)** Key population suppression drive designs that have been evaluated in the laboratory. **(C)** Population suppression drive design which targets a female fertility or female essential gene (Hammond *et al*., 2016; Carrami *et al*., 2018; Kyrou *et al*., 2018; Oberhofer *et al*., 2018). The example shown is similar to a suppression approach used in a gene drive built in *An. gambiae* (Kyrou *et al*., 2018). This drive was designed to target a female-specific transcript of the doublesex (*dsx*) gene, which is involved in sex determination. **(D)** A sex distorter gene drive was engineered in *An. gambiae*, which coupled a homing drive similar to the example in **(C)** and a sex distorter, IPpol, that destroys the X-chromosome (Simoni *et al*., 2020). **(E)** A tethered toxin-antidote recessive embryo (TARE) system was developed in *D. melanogaster* using a confinable TARE system, which facilitates confinable, homing independent inheritance of the drive (Metzloff *et al*., 2022). TARE and cleave-and-rescue (ClvR) drives **(J)** rely upon maternal carryover of Cas9 and gRNAs targeting a gene essential for development and a recoded target sequence to rescue the function of the target gene to bias inheritance. (**E** bottom) A split homing suppression gene drive with multiplexed gRNAs (Yang *et al*., 2022). **(F–J)** Key population modification drive designs that have been developed to date. **(F)** Multiple early iterations of a population modification drive targeted a non-essential gene (Gantz *et al*., 2015; Champer *et al*., 2019b). These gene drives were plagued by the rapid accumulation of cleavage resistance alleles, due to non-homologous end joining (NHEJ) because of maternal carryover of Cas9 and gRNAs. To address drive resistance, additional designs aimed to reduce the accumulation of drive resistant alleles **(G–I)**. **(G)** Homing gene drives with a rescue targeting a non-essential gene can retain

to gRNAs targeting an eye pigment gene, *cardinal*, and a transformation marker. The drive spread to fixation in small population studies in as little as four to six generations at a 1:1 release ratio. Drive resistance did develop in these populations; consequently, it is unknown how long the drive would persist in the population. The evolution of resistance to the drive is a central problem in the development of homing gene drives (Burt, 2003; Deredec *et al*., 2008; Esvelt *et al*., 2014; Bull, 2016; Callaway, 2017; Eckhoff *et al*., 2017; Noble *et al*., 2017; Unckless, *et al*., 2017) and researchers are now focusing on ways to avert the development of drive resistance. Another homing linked drive system, engineered in the Asian malaria vector *An. stephensi*, aimed to circumvent the development of drive resistance by designing a homing gene drive, *Reckh,* to target *kynurenine hydroxylase* (*kh*), a gene required for female survival and reproduction (Adolfi *et al*., 2020). If a mutation, insertion, or deletion is developed in any of the required target sites in the gene, such as the protospacer adjacent motif (PAM) site or the cleavage site, offspring inheriting non-functional resistant alleles should have a lower fitness, as the target gene is required for viable females. A recoded copy of the gene is also included in the drive to retain the function of the target gene. Notwithstanding, functional resistant mutations were still identified at low rates in the laboratory population, but did not prevent the introduction of the drive and were not predicted to outcompete the drive in small-population

cage experiments. However, whether this will hold true in large, wild, diverse populations and when linked to a gene or genes to confer disease refractoriness is unknown.

While these homing drives are clearly a substantial advancement, they are linked (Cas9 and gRNA at the same loci) and therefore are autonomous in their spread, as their high frequency of co-occurrence due to linkage makes the gene conversion process more frequent. Regulatory and significant ethical concerns surrounding the autonomous nature of the drives will likely make it difficult to gain approval for their use in field (Brossard *et al*., 2019). Alternative designs, such as those that are reversible/controllable in their spread (e.g. homing split drives), should also be a top priority for development. This strategy will ensure that there are sufficient options for mosquito control if homing linked drives are not approved for use globally.

Homing gene drives developed for population modification contain the basic Cas9 and gRNA components, but they also are linked to a gene that creates a beneficial phenotype, such as disease refractoriness. To date, there have been a number of different natural and synthetic genes, or effectors, that have been developed to prevent the transmission of one or more pathogens. Multiple reviews contain specifics on the design and efficacy of these effectors (Carballar-Lejarazú and James, 2017; Marshall *et al*., 2019; Williams *et al*., 2020; Reid *et al*., 2021) (Bottino-Rojas and James, Chapter 11; Franz, Chapter 22, this volume), but most

---

Fig. 8.3. Continued.

target gene function and possibly reduce fitness costs associated with the drive. This is the approach taken with the development of a population modification drive for *An. gambiae* (Carballar-Lejarazú *et al*., 2020). **(H)** Homing gene drives with rescue that target a haplo-insufficient gene impart a high fitness cost, since two functional alleles are needed for viability, so somatic cleavage in the absence of homing results in lethal biallelic mosaicism (Champer *et al*., 2020b). **(I)** Home-and-rescue (HomeR) drives target a haplo-sufficient essential gene and provide a recoded cleavage-resistant version of the target gene. Precise homing restores function of the target gene and any maternal carryover-associated resistance alleles are lethal and are therefore removed from the populations (Kandul *et al*., 2021; Terradas *et al*., 2021). **(J)** Cleave-and-rescue (ClvR) drives are similar to TARE drives **(E)**, whereby multiple gRNAs are engineered to target an essential gene (Oberhofer *et al*., 2019, 2020, 2021a). This gene drive system does not rely on homing and encodes a cleavage-resistant copy of the target essential gene. A temperature-sensitive (TS) version of the ClvR system encodes a temperature-sensitive self-excising protein, known as an intein, which restores the target gene function at higher temperature, resulting in inducible lethality, and following population modification this can result in population suppression (Oberhofer *et al*., 2021b). Figure created with BioRender.com

are designed to ensure appropriate tissue specificity and timing. For example, the first point of infection for vector-borne pathogens is in the midgut; consequently, midgut-specific promoters have been effectively used to disrupt infection in the early stages of infection and dissemination. Some of these promoters are expressed constitutively, such as the anopheline midgut peritrophin (Abraham *et al.*, 2005), salivary gland-specific anopheline antiplatelet protein (Yoshida and Watanabe, 2006), or anopheline apyrase (Lombardo *et al.*, 2005) promoters and *Ae. aegypti* salivary gland-specific 30K protein (Mathur *et al.*, 2010) promoters. Others promoters are inducible upon acquisition of a bloodmeal, such as the bloodmeal-inducible carboxypeptidase (Moreira *et al.*, 2000; Ito *et al.*, 2002), trypsin (Nolan *et al.*, 2011) and G12 promoters (Nolan *et al.*, 2011) for midgut-specific transgene expression, or vitellogenin (Chen *et al.*, 2007b) for expression in the haemolymph. Recently a study used the locus of multiple endogenous genes to integrate and regulate the expression of an effector gene in *An. gambiae* and was able to convert them to non-autonomous homing split drives (Hoermann *et al.*, 2021). This innovative approach minimizes the synthetic and foreign DNA that is integrated into the target species genome and provides a new method for generating non-autonomous drives. Notwithstanding these developments, there are limited effectors developed to date that target one or more pathogens (Yen *et al.*, 2018; Buchman *et al.*, 2019a,b); and for some disease vectors, such as *Ae. aegypti*, which transmit multiple pathogens, pan-antiviral effectors will need to be developed to effectively combat multiple pathogens. The effectors to date have also only been studied against a minimal number of pathogen strains, so it is still unknown whether disease refractoriness is translatable to field conditions where pathogen populations are more heterogeneous.

While there are many unique effector designs and mechanisms, the likelihood that pathogens evolve resistance to the effector is high; so, unlike suppression drives, drives designed for population modification have the additional obstacle of potential resistance to the effector. To date, there have been no evaluations of the long-term performance of effectors in large diverse mosquito and pathogen populations, so the extent of this resistance and impact on population modification efforts are presently unknown. However, based on prevalent drug resistance seen in many human pathogens, resistance should be a major consideration in effector design (Marshall *et al.*, 2019). There have been some strategies used to prevent or slow the emergence of pathogen resistance. Multiple effectors targeting the pathogen at multiple genomic sites, or in multiple life stages (Isaacs *et al.*, 2011, 2012; Mishra *et al.*, 2016; Buchman *et al.*, 2019b) have aimed to mitigate the development of effector resistance in pathogen populations; however, without additional studies to determine how these function in diverse pathogen and vector populations, it is still unclear what impact pathogen resistance will have on the sustainability of population modification. Increasing the number of effectors may circumvent pathogen resistance to the effector, but this may also impact drive performance. Since homing gene drives with a linked effector or effectors have yet to be evaluated extensively, even in the laboratory, how multiple effectors and their design might impact drive performance remains unknown. Furthermore, more studies are needed to ensure that whatever impact the effector has on the pathogen population, it does not lead to selection for escape mutants with higher pathogen load, virulence or pathogenicity (Marshall *et al.*, 2019). As these tools potentially segue into the field, pathogen resistance monitoring and management will become paramount. Indeed, monitoring and management efforts should account for the predicted spread of the drive, with increased and more widespread monitoring needed for non-localized drives such as homing linked drives. Moving forward, the continued development of new effectors will ensure that population modification strategies remain effective for managing the disease.

8.4 Population Suppression

Population suppression technologies are another important tool for vector and pest control. For many pest species, such as

agricultural pests that cause physical damage to crops, population suppression (see Fig. 8.1D), as opposed to population replacement, is the more appropriate goal. In areas where mosquito biting activity itself has a large economic impact, such as tourist destinations, suppression technologies would also be more beneficial. Nonetheless, major impacts on disease transmission have been achieved with conventional population suppression technologies. Population suppression tools are essential to vector control programmes and can be particularly effective in combination with other control methodologies. For example, implementing a vector reduction intervention prior to the release of a population modification gene drive, termed 'suppress-then-modify', may facilitate the more rapid establishment of the desired modification in the population and this could likely be achieved with fewer releases. This suppress-then-modify approach can also provide a means to reduce disease transmission while waiting for the modification to establish at a high frequency in a population. Since the evolutionary stability of population modification gene drives with integrated effectors has also yet to be evaluated in the field, it is important that proven strategies for disease control, such as population suppression, remain at the forefront of development. In another example, gene drive-based population suppression could be implemented after a conventional population suppression method. Coupling insecticides and gene drive-based suppression technologies, for example, would not only hasten the implementation of the gene drive intervention, but it could perhaps also minimize insecticide use, thereby potentially reducing the development of insecticide resistance in the population. There are many scenarios in which homing gene drive population suppression technologies could support current or future vector control and disease management programmes, but in the end, the expansion of the population suppression toolbox is vital to ensuring that technologies exist to address vector control needs.

Most population suppression homing gene drives to date encode Cas9 and gRNA components designed to target a recessive female-essential viability or fertility gene (Kyrou *et al.*, 2018; Simoni *et al.*, 2020). The *doublesex* (*dsx*) gene, in particular, is involved in sex determination and has highly conserved sex-specific splice variants across many vector species. Kyrou *et al.* (2018) found that a homing linked drive targeting a highly conserved site in *dsx* progressively eliminated small cage laboratory populations in 7–11 generations. This approach greatly reduced the accumulation of resistance alleles. Simoni *et al.* (2020) described a suppression system targeting *dsx* but they incorporated an X-chromosome shredder nuclease, I-*Ppo*I, to bias the sex ratio towards males. In 10–14 generations this sex distortion crashed multiple small cage populations. Since the drive targeted a female-specific transcript, but biased the sex ratio towards males, mutations at the target site (functional and non-functional) were rapidly removed from the population. This design also ensures that if the I-*Ppo*I nuclease, Cas9, or gRNA activity is lost, then the other component will still retain some level of population suppression. This study demonstrated the new and innovative approaches to homing gene drive design that are currently being applied to ensure that these systems are effective and predictable. However, these technologies still need to be evaluated in the field to see if they perform in a similar manner in large diverse populations. Additionally, like the population modification technologies, autonomous linked drives may pose unacceptable risks to regulators and the public, which may hamper the advancement of these technologies to the field. Therefore, the development of unlinked homing split drive and other non-autonomous genetic population suppression technologies need to be prioritized as well, to ensure that safe and effective tools are available as soon as possible to control vector-borne disease transmission.

8.5 Additional Drive Design, Performance and Implementation Considerations

Resistance to gene drives has been demonstrated in most drives developed to date. Natural drive-resistant variants can be intrinsic

to the wild population, or they may be generated *de novo* (Haag-Liautard *et al*., 2007; Marshall *et al.,* 2017*)*. Prior evaluation of the genetic structure and target site polymorphisms in the target population can help mitigate the issue of natural drive-resistant variants. Drive-resistant alleles also develop rapidly due to the NHEJ DSB repair mechanism, which can generate germline insertions or deletions (indels), or substitutions at the DSB site (Hammond *et al*., 2017; Reed, 2017; Unckless *et al*., 2017). These indels can generate sites resistant to drive cleavage, and as these indels accumulate over time due to increased NHEJ repair events and positive selection of the resistance allele, the gene drives may become extinct in a population (Marshall *et al*., 2017). In the laboratory, there have been numerous examples of the formation and accumulation of drive-resistant alleles in only a few generations even when using small cage populations (Gantz *et al*., 2015; Hammond *et al*., 2016; Champer *et al*., 2017; Carrami *et al*., 2018; Kyrou *et al*., 2018; Kandul *et al*., 2020; Li *et al*., 2020). New gene drive designs have attempted to address the accumulation of drive-resistant alleles by utilizing more germline-restricted promoters (Champer *et* al., 2018; Hammond *et al*., 2021) (see Nolan and Hammond, Chapter 3, this volume), multiplexing drive gRNAs (Marshall *et al*., 2017; Champer *et al*., 2018; Oberhofer *et al.,* 2018; Champer *et al*., 2020b), targeting highly conserved recessive essential or fertility-associated genes (Hammond *et al*., 2016; Carrami *et al*., 2018; Kyrou *et al*., 2018; Oberhofer *et al*., 2018), or by linking one or more control strategies (Simoni *et al*., 2020). By targeting an essential or fertility gene, individuals that inherit indels that result in a non-functional gene will perish and therefore not pass their resistant genotype to the subsequent generation. *De novo* mutations in the cleavage sites of these genes would likely have the same effect. A summary of key laboratory gene drive studies in *D. melanogaster* and multiple mosquito species is shown in Table 8.1.

More recently toxin–antidote (TA)-based drives have been evaluated as an innovative means to avoid the accumulation of drive-resistant alleles in populations. In many of these systems, the drive is achieved in the absence of homing. These systems have a toxin containing multiple gRNAs targeting an essential gene and the linked antidote is a cleavage-resistant copy of the target gene (Oberhofer *et al*., 2019, 2020, 2021a; Champer *et al*., 2020a). If the antidote is not inherited, then the offspring are not viable, thereby preventing the accumulation of drive resistance and increasing drive frequency in the population. Recently, homing-based TA drives have been developed in mosquitoes (Adolfi *et al*., 2020) and flies (Champer *et al*., 2020b; Kandul *et al*., 2021; Terradas *et al*., 2021). Two of these home-and-rescue (HomeR) designs, which both target essential haplo-sufficient genes, demonstrate important design considerations for these drives such as differences in target gene selection, chromosomal location (Terradas *et al*., 2021), Cas9 promoter (Kandul *et al*., 2021; Terradas *et al*., 2021) and conservation and location of target sites (Kandul *et al*., 2021; Terradas *et al*., 2021). These studies also designed additional features, such as an exogenous 3′ UTR to prevent recombination between the drive and endogenous target to increase drive stability (Kandul *et al*., 2021). Each of these features had a positive impact on the performance and stability of the gene drive in small cage populations and demonstrated the importance of these design considerations. Notably, these were developed as homing split drive systems, where the Cas9 and gRNA are on separate loci, so that the drive is restricted to individuals who inherit both components. In the laboratory, this split design simplifies the study of the individual components, but in the field, this design may provide a safer and more confineable alternative to the homing linked drive design.

8.6   A Phased Approach to Gene Drive Advancement to the Field

The existing technical capabilities of CRISPR-based technologies allow us to propose an additional spin on the NASEM stepwise approach of advancing a single technology through a phased testing pathway (Phase 1: laboratory; Phase 2: field; Phase 3: open release; Phase 4: monitoring) (NASEM, 2016).

Instead, the main components that direct the biased inheritance of the drive can perhaps be configured to: (i) not bias inheritance and not spread into a population; (ii) bias inheritance in a localized area in a self-limiting manner; and (iii) bias inheritance in a wide area in a self-propagating manner (Fig. 8.4).

In fact, there are multiple examples of genetic systems that fit each of these criteria that could be further refined for field testing. Precision-guided sterile insect technique (pgSIT) has been developed in flies (Kandul *et al.*, 2019) and mosquitoes (Li *et al.*, 2021). The pgSIT system consists of remarkably similar components often used in a gene drive, including a germline-expressed Cas9 endonuclease and gRNAs programmed to target male fertility and female viability genes. Prior to release, the Cas9 and gRNA lines are reciprocally crossed and the resulting progeny are sterile males. Therefore, released males encode the Cas9 and gRNA components that are expressed using the same germline or pol III promoters used in gene drive technologies, but since the males are sterile, these components are not preferentially inherited, nor are they predicted to persist in the environment. Trials using pgSIT, or a similar technology that does not have the risk of spreading into a population, would be a safer first step in a more integrated stepwise approach to evaluating essential drive components in the field. While other self-limiting population suppression systems do exist, for example *Wolbachia* IIT and RIDL (see Morrison, Chapter 23, this volume) (Alphey *et al.*, 2013), pgSIT utilizes many of the same components as a gene drive and therefore may prove ideal for a stepwise component testing approach. After the risk assessment of these technologies, and assuming higher efficiency is even needed, then testing could begin on more localized drives, such as homing split drives, where these components can facilitate super-Mendelian inheritance of the drive, but in a way that is more predictable, localizable and safe. These homing split drives will enable important questions to be addressed prior to proceeding with a homing linked drive system. For example, was the homing split drive effective? Did the linked effector prevent disease transmission? Did any unintended consequences arise? If these localized drive technologies are shown to have acceptable risk and efficacy and assuming additional non-localized drives are still needed for disease control, then perhaps homing linked drives could be
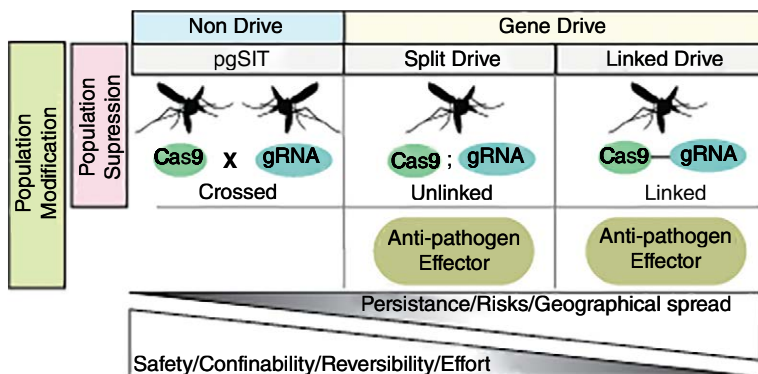
Fig. 8.4. A stepwise testing approach for both population modification and suppression. There exists an inverse relationship between various CRISPR-based population control technologies. In terms of safety, confinablity, reversibility and effort: pgSIT (highest ranked); split drive (middle ranked); linked drive (lowest ranked). Inversely, in terms of persistence, risks, geographical spread: pgSIT (lowest ranked); split drive (middle ranked); linked drive (highest ranked). To safely test various drive components (Cas9, gRNAs, transgenes, markers, effectors, etc.) perhaps a phased approach could begin with pgSIT then move to split drive, and finally, once all components have been safely tested and deemed safe, a linked drive could be the final step, assuming all ethical, safety and regulatory issues have been resolved, and if it is still necessary.

trialled on a go/no-go basis, taking into consideration all concerns articulated above. Using both a field trial stepwise (laboratory, to confined trials, to field) and a technological component stepwise approach (non-drives, to localized homing split drives, to non-localized homing linked drives) may be the optimal and safest approach (while conservative) to increasing public and regulatory acceptance of gene drive technologies. Of course, this also must be weighed against the need for these technologies, as in many cases they aim to prevent diseases that affect millions of people per year. However, it is important that we consider when and if gene drives are needed to support disease management and how these technologies should be tested and evaluated prior to and post field trial.

8.7   Concluding Remarks

Gene drive and genetic population control technologies certainly have a lot of promise, but they will not solve the issues they are built to address alone. As a vector-borne disease control tool, for example, gene drive technologies will likely be one of many technologies needed to eliminate these diseases. The complexity of the disease transmission and the local, regional and global variability of vector population dynamics, the environment and other factors make a 'one size fits all' approach to the control of these diseases simply unrealistic. This issue is further complicated by the fact that disease vectors and diseases do not adhere to political boundaries (see Pereira, Chapter 27, this volume). A disease intervention that is acceptable to the values and needs of one country may not be acceptable to a neighbouring country. The larger the intervention toolbox, the more likely it is that an affordable, acceptable and effective solution can be found that meets the needs of any area impacted by these diseases.

Gene drive technologies do have a lot of features that make them a potentially powerful tool to combat diseases. Their self-sustaining behaviour should make their implementation and maintenance costs lower than those for other interventions. This lower cost may

make this technology accessible to areas without the financial resources, infrastructure, or political stability to implement and maintain other control programmes. Disease control programmes that were interrupted by the recent Covid19 pandemic, for example, may have been able to continue if they were easier to maintain. In many areas impacted by vector-borne disease, conflict and destabilization of political and social structures also commonly impacts these programmes and could therefore also benefit from low-maintenance control technologies.

Gene drive-based interventions and other genetic control approaches would also not rely upon the behaviour of individuals to interrupt disease transmission. Many of the disease interventions currently employed in the field also require a fair amount of individual participation to maintain. Insecticide-treated bednets (ITNs), for instance, require individuals to sleep under the nets and to wash and care for them appropriately to maintain their integrity. Certainly, in recent years improved ITNs with long-lasting insecticides have made a dramatic impact on malaria transmission, but ITN compliance is still variable and often requires frequent outreach and educational efforts to ensure high compliance.

Gene drive technologies and other genetic control approaches could also ensure more equitable protection of whole communities. Everyone in the treatment areas regardless of their race, religion, political affiliation, socioeconomic status and access to educational or medical resources would equally benefit from these technologies. Anywhere they travel within the community (schools, workplaces, markets, etc.) they will be protected and individuals away from home will also have the same protection as individuals in the home. Other interventions, such as topical repellents and insecticide-treated clothing, are in development to address transmission outside the home, but these are unlikely to be affordable or accessible to all community members.

The lack of interventions for outside the home is a main deficiency in the capabilities of current mosquito control tools. ITNs and indoor residual spraying (IRS) reduce

the human-biting rate indoors only, but there are a number of malaria vectors that bite outdoors. The interventions currently available to target outdoor-biting vectors are limited and therefore at least some level of residual malaria transmission is predicted to continue even with high ITN and IRS coverage (Sherrard-Smith *et al.*, 2019). Gene drive-based interventions will work against both indoor- and outdoor-biting vectors. In fact, as control efforts for indoor-biting mosquitoes have intensified, some vectors are modifying their behaviour and increasing their outdoor biting. In situations where vectors have these dynamic responses to conventional interventions, gene drive-based interventions may also be able to address disease transmission from outdoor-biting vectors. Additionally, as insecticide resistance becomes more prevalent and conventional control measures fail, alternative interventions, such as gene drives, need to be in the pipeline to support control efforts.

The species specificity of gene drive technology is assuredly an environmental benefit, as only the target organism is affected. Insecticide-based interventions, in contrast, have indiscriminate impacts on many species, including beneficial species. Species specificity is a double-edged sword, however, for pathogens with multiple vectors. In many areas, malaria is transmitted by multiple species, literally a complex of species, so to achieve sustained disease control it may unfortunately be necessary to develop and release a separate gene drive for each vector species. Currently, there are limited resources and genetic tools for multiple primary malaria vectors, such as *Anopheles arabiensis*, *Anopheles funestus*, *Anopheles moucheti* and *Anopheles nili*, so significant investment is needed in studying these vectors, if gene drive systems are to be an effective strategy to combat malaria globally. There are also numerous secondary malaria vectors that typically only account for 5% of the disease transmission (Afrane, 2016), but in some cases can potentiate malaria transmission in the absence of the primary vectors (Gillies and Smith, 1960; Afrane, 2016). Consequently, as primary vectors are eradicated, malaria transmission may continue. Additional considerations and modelling for this potential will need to be evaluated when implementing disease management strategies.

The gene drive research community has been more proactive than most emerging technologies in developing guidelines for safe and responsible technological development. Shortly after the development of the first CRISPR-based linked homing gene drive in mosquitoes (Gantz *et al.*, 2015), guidance on the safe development of gene drives in the laboratory was published and co-authored by key leaders in the field (Akbari *et al.*, 2015). These guidelines outlined safety measures for researchers to ensure that these drives stay confined to the laboratory during development. As gene drive technology becomes closer to potential field release, more self-governance has been developed by leaders in the field that outlines the minimal core commitments for field trials of gene drive organisms (Long *et al.*, 2020). These include pledges towards engagement, scientific integrity and public transparency in gene drive trials. The four main core commitments are as follows.

1. Fair partnership and transparency by engaging stakeholders in the trial design to improve quality and accountability as well as a commitment to involve stakeholders in the ongoing analytical trial assessments and to open and timely sharing of trial data with stakeholders.

2. Product efficacy and safety, which involves setting acceptable performance standards through collaboration from regulators and other stakeholders, identifying uncertainties in safety and efficacy and ensuring that the efficacy and safety data are made publicly available.

3. Regulatory evaluation and risk/benefit assessment, which includes early and frequent engagement with the appropriate international, national and regional authorities for ethics and regulatory approvals, while developing methods to assess the benefits of these technologies and expanding these assessments to be more inclusive of expertise from communities and other stakeholders.

4. Monitoring and mitigation, focusing on the engagement of regulators and stakeholders during the development of mitigation and monitoring plans, which include defining when and how monitoring and mitigation efforts should be conducted and timely reporting of the safety and efficacy field data.

Again, many influential leaders in the gene drive field co-signed this guidance and have pledged to follow this guidance as their technologies move forward in development. These efforts underscore that the research community recognizes the potential risks of these technologies, but also understands the importance of ensuring that these possibly ground-breaking technologies can safely continue to advance with the ultimate goal of reducing our reliance on harmful chemicals while solving some of the world's most daunting global health problems.

Acknowledgements

References

Abraham, E.G., Donnelly-Doman, M., Fujioka, H., Ghosh, A., Moreira, L. and Jacobs-Lorena, M. (2005) Driving midgut-specific expression and secretion of a foreign protein in transgenic mosquitoes with AgAper1 regulatory elements. *Insect Molecular Biology* 14, 271–279.

Adolfi, A., Gantz, V.M., Jasinskiene, N., Lee, H.-F., Hwang, K. *et al.* (2020) Efficient population modification gene-drive rescue system in the malaria mosquito *Anopheles stephensi*. *Nature Communications* 11, 5553.

Afrane, Y.A. (2016) Secondary malaria vectors of Sub-Saharan Africa: threat to malaria elimination on the continent? In: Rodriguez-Morales, A.J. (ed.) *Current Topics in Malaria*. IntechOpen, London. doi: 10.5772/65359.

Akbari, O.S., Matzen, K.D., Marshall, J.M., Huang, H., Ward, C.M. and Hay, B.A. (2013) A synthetic gene drive system for local, reversible modification and suppression of insect populations. *Current Biology* 23, 671–677.

Akbari, O.S., Chen, C.-H., Marshall, J.M., Huang, H., Antoshechkin, I. and Hay, B.A. (2014) Novel synthetic *Medea* selfish genetic elements drive population replacement in *Drosophila*; a theoretical exploration of *Medea*-dependent population suppression. *ACS Synthetic Biology* 3, 915–928.

Akbari, O.S., Bellen, H.J., Bier, E., Bullock, S.L., Burt, A. *et al.* (2015) BIOSAFETY. Safeguarding gene drive experiments in the laboratory. *Science* 349, 927–929.

Alphey, L., McKemey, A., Nimmo, D., Neira Oviedo, M., Lacroix, R. *et al.* (2013) Genetic control of *Aedes* mosquitoes. *Pathogens and Global Health* 107, 170–179.

Arrighi, R.B.G., Ebikeme, C., Jiang, Y., Ranford-Cartwright, L., Barrett, M.P. *et al.* (2008) Cell-penetrating peptide TP10 shows broad-spectrum activity against both *Plasmodium falciparum* and *Trypanosoma brucei brucei*. *Antimicrobial Agents and Chemotherapy* 52, 3414–3417.

Asman, S.M., McDonald, P.T. and Prout, T. (1981) Field studies of genetic control systems for mosquitoes. *Annual Review of Entomology* 26, 289–318.

Barreau, C., Touray, M., Pimenta, P.F., Miller, L.H. and Vernick, K.D. (1995) *Plasmodium gallinaceum*: sporozoite invasion of *Aedes aegypti* salivary glands is inhibited by anti-gland antibodies and by lectins. *Experimental Parasitology* 81, 332–343.

Bhat, K.P. and Cortez, D. (2018) RPA and RAD51: fork reversal, fork protection, and genome stability. *Nature Structural & Molecular Biology* 25, 446–453.

Branzei, D. and Foiani, M. (2008) Regulation of DNA repair throughout the cell cycle. *Nature Reviews Molecular Cell Biology* 9, 297–308.

Brossard, D., Belluck, P., Gould, F. and Wirz, C.D. (2019) Promises and perils of gene drives: Navigating the communication of complex, post-normal science. *Proceedings of the National Academy of Sciences USA* 116, 7692–7697.

Buchman, A.B., Ivy, T., Marshall, J.M., Akbari, O.S. and Hay, B.A. (2018a) Engineered reciprocal chromosome translocations drive high threshold, reversible population replacement in *Drosophila*. *ACS Synthetic Biology* 7, 1359–1370.

Buchman, A., Marshall, J.M., Ostrovski, D., Yang, T. and Akbari, O.S. (2018b) Synthetically engineered *Medea* gene drive system in the worldwide crop pest *Drosophila suzukii*. *Proceedings of the National Academy of Sciences USA* 115, 4725–4730.

Buchman, A., Gamez, S., Li, M., Antoshechkin, I., Li, H.-H. *et al.* (2019a) Broad dengue neutralization in mosquitoes expressing an engineered antibody. *PLoS Pathogens* 16, e1008103.

Buchman, A., Gamez, S., Li, M., Antoshechkin, I., Li, H.-H. *et al.* (2019b) Engineered resistance to Zika virus in transgenic *Aedes aegypti* expressing a polycistronic cluster of synthetic small RNAs. *Proceedings of the National Academy of Sciences USA* 116, 3656-3661.

Buchman, A., Shriner, I., Yang, T., Liu, J., Antoshechkin, I. *et al.* (2020) Engineered reproductively isolated species drive reversible population replacement. *Nature Communications* 12, 3281.

Bull, J.J. (2016) Lethal gene drive selects inbreeding. *Evolution, Medicine, and Public Health* 2017, 1–16.

Burt, A. (2003) Site-specific selfish genes as tools for the control and genetic engineering of natural populations. *Proceedings of the Royal Society B: Biological Sciences* 270, 921–928.

Callaway, E. (2017) Gene drives meet the resistance. *Nature* 542, 15–15.

Carballal-Lejarazú, R. and James, A.A. (2017) Population modification of anopheline species to control malaria transmission. *Pathogens and Global Health* 111, 424–435.

Carballal-Lejarazú, R., Rodríguez, M.H., de la Cruz Hernández-Hernández, F., Ramos-Castañeda, J., Possani, L.D. *et al.* (2008) Recombinant scorpine: a multifunctional antimicrobial peptide with activity against different pathogens. *Cellular and Molecular Life Sciences* 65, 3081–3092.

Carballal-Lejarazú, R., Ogaugwu, C., Tushar, T., Kelsey, A., Pham, T.B. *et al.* (2020) Next-generation gene drive for population modification of the malaria vector mosquito, *Anopheles gambiae*. *Proceedings of the National Academy of Sciences USA* 117, 22805–22814.

Carrami, E.M., Eckermann, K.N., Ahmed, H.M.M., Sánchez Castellanos, H.M., Dippel, S. *et al.* (2018) Consequences of resistance evolution in a Cas9-based sex conversion-suppression gene drive for insect pest management. *Proceedings of the National Academy of Sciences USA* 115, 6189–6194.

Carter, J.R., Taylor, S., Fraser, T.S., Kucharski, C.A., Dawson, J.L. and Fraser, M.J. Jr (2015) Suppression of the arboviruses dengue and chikungunya using a dual-acting group-I intron coupled with conditional expression of the Bax C-terminal domain. *PloS ONE* 10, e0139899.

Champer, J., Buchman, A. and Akbari, O.S. (2016) Cheating evolution: engineering gene drives to manipulate the fate of wild populations. *Nature Reviews Genetics* 17, 146–159.

Champer, J., Reeves, R., Oh, S.Y., Liu, C., Liu, J. *et al.* (2017) Novel CRISPR/Cas9 gene drive constructs reveal insights into mechanisms of resistance allele formation and drive efficiency in genetically diverse populations. *PLoS Genetics* 13, e1006796.

Champer, J., Liu, J., Oh, S.Y., Reeves, R., Luthra, A. *et al.* (2018) Reducing resistance allele formation in CRISPR gene drive. *Proceedings of the National Academy of Sciences USA* 115, 5522–5527.

Champer, J., Chung, J., Lee, Y.L., Liu, C., Yang, E. *et al.* (2019b) Molecular safeguarding of CRISPR gene drive experiments. *eLife* 8, e41439.

Champer, J., Wen, Z., Luthra, A., Reeves, R., Chung, J. *et al.* (2019a) CRISPR gene drive efficiency and resistance rate is highly heritable with no common genetic loci of large effect. *Genetics* 212, 333–341.

Champer, J., Lee, E., Yang, E., Liu, C., Clark, A.G. and Messer, P.W. (2020a) A toxin-antidote CRISPR gene drive system for regional population modification. *Nature Communications* 11, 1082.

Champer, J., Yang, E., Lee, E., Liu, J., Clark, A.G. and Messer, P.W. (2020b) A CRISPR homing gene drive targeting a haplolethal gene removes resistance alleles and successfully spreads through a cage population. *Proceedings of the National Academy of Sciences USA* 117, 24377–24383.

Champer, J., Kim, I.K., Champer, S.E., Clark, A.G. and Messer, P.W. (2021) Suppression gene drive in continuous space can result in unstable persistence of both drive and wild-type alleles. *Molecular Ecology* 30, 1086–1101.

Chen, C.-H., Huang, H., Ward, C.M., Su, J.T., Schaeffer, L.V. *et al.* (2007a) A synthetic maternal-effect selfish genetic element drives population replacement in *Drosophila*. *Science* 316, 597–600.

Chen, X.-G., Marinotti, O., Whitman, L., Jasinskiene, N., James, A.A. and Romans, P. (2007b) The *Anopheles gambiae* vitellogenin gene (VGT2) promoter directs persistent accumulation of a reporter gene product in transgenic *Anopheles stephensi* following multiple bloodmeals. *The American Journal of Tropical Medicine and Hygiene* 76, 1118–1124.

Conde, R., Zamudio, F.Z., Rodríguez, M.H. and Possani, L.D. (2000) Scorpine, an anti-malaria and anti-bacterial agent purified from scorpion venom. *FEBS Letters* 471,165–168.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R. *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823.

Corby-Harris, V., Drexler, A., de Jong, L.W., Antonova, Y., Pakpour, N. *et al.* (2010) Activation of akt signaling reduces the prevalence and intensity of malaria parasite infection and lifespan in *Anopheles stephensi* mosquitoes. *PLoS Pathogens* 6, e1001003.

Curtis, C.F. (1968) Possible use of translocations to fix desirable genes in insect pest populations. *Nature* 218, 368–369.

Curtis, C.F., Southern, D.I., Pell, P.E. and Craig-Cameron, T.A. (1972) Chromosome translocations in *Glossina austeni*. *Genetics Research* 20, 101–113.

de Lara Capurro, M., Coleman, J., Beerntsen, B.T., Myles, K.M., Olson, K.E. *et al.* (2000) Virus-expressed, recombinant single-chain antibody blocks sporozoite infection of salivary glands in *Plasmodium gallinaceum*-infected *Aedes aegypti*. *The American Journal of Tropical Medicine and Hygiene* 62, 427–433.

Deredec, A., Burt, A. and Godfray, H.C.J. (2008) The population genetics of using homing endonuclease genes in vector and pest management. *Genetics* 179, 2013–2026.

Dong, Y., Das, S., Cirimotich, C., Souza-Neto, J.A., McLean, K.J. and Dimopoulos, G. (2011) Engineered *anopheles* immunity to *Plasmodium* infection. *PLoS Pathogens* 7, e1002458.

Eckhoff, P.A., Wenger, E.A., Godfray, H.C.J. and Burt, A. (2017) Impact of mosquito gene drive on malaria elimination in a computational model with explicit spatial and temporal dynamics. *Proceedings of the National Academy of Sciences USA* 114, E255–E264.

Esvelt, K.M., Smidler, A.L., Catteruccia, F. and Church, G.M. (2014) Concerning RNA-guided gene drives for the alteration of wild populations. *eLife* 3, e03401.

Fang, W., Vega-Rodríguez, J., Ghosh, A.K., Jacobs-Lorena, M., Kang, A. and St Leger, R.J. (2011) Development of transgenic fungi that kill human malaria parasites in mosquitoes. *Science* 331, 1074–1077.

Franz, A.W.E., Sanchez-Vargas, I., Adelman, Z.N., Blair, C.D., Beaty, B.J. *et al.* (2006) Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. *Proceedings of the National Academy of Sciences USA* 103, 4198–4203.

Franz, A.W.E., Sanchez-Vargas, I., Raban, R.R., Black, W.C. 4th, James, A.A. and Olson, K.E. (2014) Fitness impact and stability of a transgene conferring resistance to dengue-2 virus following introgression into a genetically diverse *Aedes aegypti* strain. *PLoS Neglected Tropical Diseases*, 8, e2833.

Galizi, R., Hammond, A., Kyrou, K., Taxiarchi, C., Bernardini, F., *et al.* (2016) A CRISPR-Cas9 sex-ratio distortion system for genetic control. *Scientific Reports* 6, 31139.

Gantz, V.M. and Bier, E. (2015) The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. *Science* 348, 442–444.

Gantz, V.M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V.M. *et al.* (2015) Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. *Proceedings of the National Academy of Sciences USA* 112, E6736–E6743.

Gao, B., del Carmen Rodriguez, M., Lanz-Mendoza, H. and Zhu, S. (2009) AdDLP, a bacterial defensin-like peptide, exhibits anti-*Plasmodium* activity. *Biochemical and Biophysical Research Communications* 387, 393–398.

Gao, B., Xu, J., Rodriguez, M. del C., Lanz-Mendoza, H., Hernández-Rivas, R *et al.* (2010) Characterization of two linear cationic antimalarial peptides in the scorpion *Mesobuthus eupeus*. *Biochimie* 92, 350–359.

Gillies, M.T. and Smith, A. (1960) The effect of a residual house-spraying campaign in East Africa on species balance in the *Anopheles funestus* group. The replacement of *A. funestus* Giles by *A. rivulorum* Leeson. *Bulletin of Entomological Research* 51, 243–252.

Gwadz, R.W., Kaslow, D., Lee, J.Y., Maloy, W.L., Zasloff, M. and Miller, L.H. (1989) Effects of magainins and cecropins on the sporogonic development of malaria parasites in mosquitoes. *Infection and Immunity* 57, 2628–2633.

Haag-Liautard, C., Dorris, M., Maside, X., Macaskill, S., Halligan, D. L. *et al.* (2007) Direct estimation of per nucleotide and genomic deleterious mutation rates in *Drosophila*. *Nature* 445, 82–85.

Hamilton, W.D. (1967) Extraordinary sex ratios. A sex-ratio theory for sex linkage and inbreeding has new implications in cytogenetics and entomology. *Science* 156, 477–488.

Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C. *et al.* (2016) A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nature Biotechnology* 34, 78–83.

Hammond, A.M., Kyrou, K., Bruttini, M., North, A., Galizi, R. *et al.* (2017) The creation and selection of mutations resistant to a gene drive over multiple generations in the malaria mosquito. *PLoS Genetics* 13, e1007039.

Hammond, A., Karlsson, X., Morianou, I., Kyrou, K., Beaghton, A., Gribble, M., Kranjc, N., Galizi, R., Burt, A., Crisanti, A., Nolan, T. (2021) Regulating the expression of gene drives is key to increasing their invasive potential and the mitigation of resistance. PLoS Genetics 17, e1009321.

Heyer, W.-D., Ehmsen, K.T. and Liu, J. (2010) Regulation of homologous recombination in eukaryotes. *Annual Review of Genetics* 44, 113–139.

Hoermann, A., Tapanelli, S., Capriotti, P., Del Corsano, G., Masters, E.K. *et al.* (2021) Converting endogenous genes of the malaria mosquito into simple non-autonomous gene drives for population replacement. *eLife* 10, e58791.

Isaacs, A.T., Li, F., Jasinskiene, N., Chen, X., Nirmala, X. *et al.* (2011) Engineered resistance to *Plasmodium falciparum* development in transgenic *Anopheles stephensi*. *PLoS Pathogens* 7, e1002017.

Isaacs, A.T., Jasinskiene, N., Tretiakov, M., Thiery, I., Zettor, A. *et al.* (2012) Transgenic *Anopheles stephensi* coexpressing single-chain antibodies resist *Plasmodium falciparum* development. *Proceedings of the National Academy of Sciences USA* 109, E1922–E1930.

Ito, J., Ghosh, A., Moreira, L.A., Wimmer, E.A. and Jacobs-Lorena, M. (2002) Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature* 417, 452–455.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821.

Kandul, N.P., Liu, J., Sanchez Castellanos, H.M., Wu, S.L., Marshall, J.M. and Akbari, O.S. (2019) Transforming insect population control with precision guided sterile males with demonstration in flies. *Nature Communications* 10, 84.

Kandul, N.P., Liu, J., Buchman, A., Gantz, V.M., Bier, E. and Akbari, O.S. (2020) Assessment of a split homing based gene drive for efficient knockout of multiple genes. *G3: Genes, Genomics, and Genetics* 10, 827–837.

Kandul, N.P., Liu, J., Bennett, J.B., Marshall, J.M. and Akbari, O.S. (2021) A confinable home-and-rescue gene drive for population modification. *eLife* 10, e65939.

Kowalczykowski, S.C. (2015) An overview of the molecular mechanisms of recombinational DNA repair. *Cold Spring Harbor Perspectives in Biology* 7, a016410.

Kyrou, K., Hammond, A.M., Galizi, R., Kranjc, N., Burt, A. *et al.* (2018) A CRISPR–Cas9 gene drive targeting doublesex causes complete population suppression in caged *Anopheles gambiae* mosquitoes. *Nature Biotechnology* 36, 1062.

Li, M., Yang, T., Kandul, N.P., Bui, M., Gamez, S. *et al.* (2020) Development of a confinable gene drive system in the human disease vector *Aedes aegypti.* eLife 9, 51701.

Li, M., Yang, T., Bui, M., Gamez, S., Wise, T. *et al.* (2021) Suppressing mosquito populations with precision guided sterile males. *Nature Communications* 12, 5374.

Lombardo, F., Nolan, T., Lycett, G., Lanfrancotti, A., Stich, N. *et al.* (2005) An *Anopheles gambiae* salivary gland promoter analysis in *Drosophila melanogaster* and *Anopheles stephensi*. *Insect Molecular Biology* 14, 207–216.

Long, K.C., Alphey, L., Annas, G.J., Bloss, C.S., Campbell, K.J. *et al.* (2020) Core commitments for field trials of gene drive organisms. *Science* 370, 1417–1419.

Lorimer, N., Hallinan, E. and Rai, K.S. (1972) Translocation homozygotes in the yellow fever mosquito, *Aedes aegypti*. *The Journal of Heredity* 63, 158–166.

Luckhart, S., Vodovotz, Y., Cui, L. and Rosenberg, R. (1998) The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. *Proceedings of the National Academy of Sciences USA* 95, 5700–5705.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M. *et al.* (2013) RNA-guided human genome engineering via Cas9. *Science* 339, 823–826.

Marshall, J.M. and Akbari, O.S. (2018) Can CRISPR-based gene drive be confined in the wild? A question for molecular and population biology. *ACS Chemical Biology* 13, 424–430.

Marshall, J.M., Buchman, A., Sánchez Castellanos, H.M. and Akbari, O.S. (2017) Overcoming evolved resistance to population-suppressing homing-based gene drives. *Scientific Reports* 7, 3776.

Marshall, J.M., Raban, R.R., Kandul, N.P., Edula, J.R., León, T.M. and Akbari, O.S. (2019) Winning the tug-of-war between effector gene design and pathogen evolution in vector population replacement strategies. *Frontiers in Genetics* 10, 1072.

Maselko, M., Feltman, N., Upadhyay, A., Hayward, A., Das, S. *et al.* (2020) Engineering multiple species-like genetic incompatibilities in insects. *Nature Communications* 11, 4468.

Mathur, G., Sanchez-Vargas, I., Alvarez, D., Olson, K.E., Marinotti, O. and James, A.A. (2010) Transgene-mediated suppression of dengue viruses in the salivary glands of the yellow fever mosquito, *Aedes aegypti*. *Insect Molecular Biology* 19, 753–763.

Metzloff, M., Yang, E., Dhole, S., Clark, A.G., Messer, P.W., Champer, J. (2022) Experimental demonstration of tethered gene drive systems for confined population modification or suppression. BMC Biology 20, 119.

Mishra, P., Furey, C., Balaraman, V. and Fraser, M.J. (2016) Antiviral hammerhead ribozymes are effective for developing transgenic suppression of chikungunya virus in *Aedes aegypti* mosquitoes. *Viruses* 8, 6.

Moreira, C.K., Rodrigues, F.G., Ghosh, A., Varotti, F. de P., Miranda, A. *et al.* (2007) Effect of the antimicrobial peptide gomesin against different life stages of *Plasmodium* spp. *Experimental Parasitology* 116, 346–353.

Moreira, L.A., Edwards, M.J., Adhami, F., Jasinskiene, N., James, A.A. and Jacobs-Lorena, M. (2000) Robust gut-specific gene expression in transgenic *Aedes aegypti* mosquitoes. *Proceedings of the National Academy of Sciences USA* 97, 10895–10898.

Moreira, L.A., Ito, J., Ghosh, A., Devenport, M., Zieler, H. *et al.* (2002) Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes. *The Journal of Biological Chemistry* 277, 40839–40843.

NASEM (National Academies of Sciences, Engineering and Medicine) (2016) *Gene Drives on the Horizon Advancing Science, Navigating Uncertainty, and Aligning Research with Public Values*. The National Academies Press, Washington, DC.

Noble, C., Olejarz, J., Esvelt, K.M., Church, G.M. and Nowak, M.A. (2017) Evolutionary dynamics of CRISPR gene drives. *Science Advances* 3, e1601964.

Noble, C., Adlam, B., Church, G.M., Esvelt, K.M. and Nowak, M.A. (2018) *Current CRISPR gene drive systems are likely to be highly invasive in wild populations.* eLife 7, 33423.

Nolan, T., Petris, E., Müller, H.-M., Cronin, A., Catteruccia, F. and Crisanti, A. (2011) Analysis of two novel midgut-specific promoters driving transgene expression in *Anopheles stephensi* mosquitoes. *PloS ONE* 6, e16471.

Oberhofer, G., Ivy, T. and Hay, B.A. (2018) Behavior of homing endonuclease gene drives targeting genes required for viability or female fertility with multiplexed guide RNAs. *Proceedings of the National Academy of Sciences USA* 115, E9343–E9352.

Oberhofer, G., Ivy, T. and Hay, B.A. (2019) Cleave and Rescue, a novel selfish genetic element and general strategy for gene drive. *Proceedings of the National Academy of Sciences USA* 116, 6250–6259.

Oberhofer, G., Ivy, T. and Hay, B.A. (2020) Gene drive and resilience through renewal with next generation Cleave and Rescue selfish genetic elements. *Proceedings of the National Academy of Sciences USA* 117, 9013–9021.

Oberhofer, G., Ivy, T. and Hay, B.A. (2021a). Split versions of *Cleave and Rescue* selfish genetic elements for measured self limiting gene drive. *PLoS Genetics* 17, e1009385. 1

Oberhofer, G., Ivy, T., Hay, BA. (2021b) Gene drive that results in addiction to a temperature-sensitive version of an essential gene triggers population collapse in *Drosophila*. *Proceedings of the National Academy of Sciences US* 118, e2107413118.

Papathanos, P.A., Windbichler, N. and Akbari, O.S. (2014) Sex ratio manipulation for insect population control. In: Benedict, M. (ed.) *Transgenic Insects: Techniques and Applications.* CABI Publishing, Wallingford, UK, pp. 83–100.

Raban, R.R., Marshall, J.M. and Akbari, O.S. (2020) Progress towards engineering gene drives for population control. *The Journal of Experimental Biology* 223, jeb208181.

Reed, F.A. (2017) CRISPR/Cas9 gene drive: growing pains for a new technology. *Genetics* 205, 1037–1039.

Reid, W.R., Olson, K.E. and Franz, A.W.E. (2021) Current effector and gene-drive developments to engineer arbovirus-resistant *Aedes aegypti* (Diptera: Culicidae) for a sustainable population replacement strategy in the field. *Journal of Medical Entomology* 58, 1987–1996.

Renkawitz, J., Lademann, C.A. and Jentsch, S. (2014) Mechanisms and principles of homology search during recombination. *Nature Reviews Molecular Cell Biology* 15, 369–383.

Robinson, A.S. (1976) Progress in the use of chromosomal translocations for the control of insect pests. *Biological Reviews of the Cambridge Philosophical Society* 51, 1–24.

Rodriguez, M.C., Zamudio, F., Torres, J.A., Gonzalez-Ceron, L., Possani, L.D. and Rodriguez, M.H. (1995) Effect of a cecropin-like synthetic peptide (Shiva-3) on the sporogonic development of *Plasmodium berghei*. *Experimental Parasitology* 80(4), 596–604.

Sandler, L. and Novitski, E. (1957) Meiotic drive as an evolutionary force. *The American Naturalist* 91, 105–110.

San Filippo, J., Sung, P. and Klein, H. (2008) Mechanism of eukaryotic homologous recombination. *Annual Review of Biochemistry* 77, 229–257.

Serebrovsky, A.S. *et al.* (1969) On the possibility of a new method for the control of insect pests. Sterile-male technique for eradication or control of harmful insects. *Proceedings of a Panel on Application of the Sterile-Male Technique for the Eradication or Control of Harmful Species of Insects*, organized by the Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture, Vienna, Austria, pp. 123–237.

Shahabuddin, M., Fields, I., Bulet, P., Hoffmann, J.A. and Miller, L.H. (1998) *Plasmodium gallinaceum*: differential killing of some mosquito stages of the parasite by insect defensin. *Experimental Parasitology* 89, 103–112.

Sherrard-Smith, E., Skarp, J.E., Beale, A.D., Fornadel, C., Norris, L.C. *et al.* (2019) Mosquito feeding behavior and how it influences residual malaria transmission across Africa. *Proceedings of the National Academy of Sciences USA* 116, 15086–15095.

Simoni, A., Hammond, A.M., Beaghton, A.K., Galizi, R., Taxiarchi, C. *et al.* (2020) A male-biased sex-distorter gene drive for the human malaria vector *Anopheles gambiae*. *Nature Biotechnology* 38, 1054–1060.

Symington, L.S. (2016) Mechanism and regulation of DNA end resection in eukaryotes. *Critical Reviews in Biochemistry and Molecular Biology* 51, 195–212.

Symington, L.S. and Gautier, J. (2011) Double-strand break end resection and repair pathway choice. *Annual Review of Genetics* 45, 247–271.

Terradas, G., Buchman, A.B., Bennett, J.B., Shriner, I., Marshall, J.M. *et al.* (2021) Inherently confinable split-drive systems in *Drosophila*. *Nature Communications* 12, 1480.

Tsai, Y.L., Hayward, R.E., Langer, R.C., Fidock, D.A. and Vinetz, J.M. (2001) Disruption of *Plasmodium falciparum* chitinase markedly impairs parasite invasion of mosquito midgut. *Infection and Immunity* 69, 4048–4054.

Unckless, R.L., Clark, A.G. and Messer, P.W. (2017) Evolution of resistance against CRISPR/Cas9 gene drive. *Genetics* 205, 827–841.

Verkuijl, S.A.N., Gonzalez, E., Li, M., Ang, J., Kandul, N.P. *et al.* (2020) A CRISPR endonuclease gene drive reveals two distinct mechanisms of inheritance bias. *bioRxiv*, 2020.12.15.421271.

Vizioli, J., Bulet, P., Hoffmann, J.A., Kafatos, F.C., Müller, H.M. and Dimopoulos, G. (2001) Gambicin: a novel immune responsive antimicrobial peptide from the malaria vector *Anopheles gambiae*. *Proceedings of the National Academy of Sciences USA* 98, 12630–12635.

Webster, S.H., Vella, M.R. and Scott, M.J. (2020) Development and testing of a novel killer–rescue self-limiting gene drive system in *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences* 287, 20192994.

West, S.C. (2009) The search for a human Holliday junction resolvase. *Biochemical Society Transactions* 37, 519–526.

Williams, A.E., Franz, A.W.E., Reid, W.R. and Olson, K.E. (2020) Antiviral effectors and gene drive strategies for mosquito population suppression or replacement to mitigate arbovirus transmission by *Aedes aegypti*. *Insects* 11, 52.

Yang, E., Metzloff, M., Langmüller, A.M., Xu, X., Clark, A.G., Messer, P.W., Champer J. (2022) A homing suppression gene drive with multiplexed gRNAs maintains high drive conversion efficiency and avoids functional resistance alleles. G3 12, jkac081.

Yang, H., Ren, S., Yu, S., Pan, H., Li, T. *et al.* (2020) Methods favoring homology-directed repair choice in response to CRISPR/Cas9 induced-double strand breaks. *International Journal of Molecular Sciences* 21, 18.

Yen, P.-S., James, A., Li, J.-C., Chen, C.-H. and Failloux, A.-B. (2018) Synthetic miRNAs induce dual arboviral-resistance phenotypes in the vector mosquito *Aedes aegypti*. *Communications Biology* 1, 11.

Yoshida, S. and Watanabe, H. (2006) Robust salivary gland-specific transgene expression in *Anopheles stephensi* mosquito. *Insect Molecular Biology* 15, 403–410.

Yoshida, S., Matsuoka, H., Luo, E., Iwai, K., Arai, M. *et al.* (1999) A single-chain antibody fragment specific for the *Plasmodium berghei* ookinete protein Pbs21 confers transmission blockade in the mosquito midgut. *Molecular and Biochemical Parasitology* 104, 195–204.

Yoshida, S., Ioka, D., Matsuoka, H., Endo, H. and Ishii, A. (2001) Bacteria expressing single-chain immunotoxin inhibit malaria parasite development in mosquitoes. *Molecular and Biochemical Parasitology* 113, 89–96.

Yoshida, S., Shimada, Y., Kondoh, D., Kouzuma, Y., Ghosh, A.K. *et al.* (2007) Hemolytic C-type lectin CEL-III from sea cucumber expressed in transgenic mosquitoes impairs malaria parasite development. *PLoS Pathogens* 3, e192.

Zhang, G., Niu, G., Franca, C.M., Dong, Y., Wang, X. *et al.* (2015) *Anopheles* midgut FREP1 mediates *Plasmodium* invasion. *Journal of Biological Chemistry* 290, 16490–16501.

Zieler, H., Keister, D.B., Dvorak, J.A. and Ribeiro, J.M.C. (2001) A snake venom phospholipase A2 blocks malaria parasite development in the mosquito midgut by inhibiting ookinete association with the midgut surface. *The Journal of Experimental Biology* 204, 4157–4167.

9 *Drosophila melanogaster* as a Model for Gene Drive Systems

Jackson Champer*

*Center for Bioinformatics, School of Life Sciences, Peking-Tsinghua Center
for Life Sciences, Peking University, Beijing, China 100871*

9.1 Introduction

Engineered gene drive alleles are designed to bias inheritance, increasing their frequency in a population (Fig. 9.1) (Esvelt *et al*., 2014; Bull, 2015; Gantz and Bier, 2015a; Champer *et al*., 2016; Carballar-Lejarazú and James, 2017; Macias *et al*., 2017; Burt and Crisanti, 2018; Leftwich *et al*., 2018; Quinn and Nolan, 2020; Hay *et al*., 2021). If their efficiency is sufficiently high, such drives could be used for modification or suppression by releasing a few individuals into natural populations to initiate the spread of the drive allele. Potential applications for gene drives are broad and include preventing disease transmission in mosquitoes or other vectors, often through use of attached 'payload' or 'cargo' genes that serve as the effector component of a drive. They could also be used to suppress populations such as invasive species where they are threatening ecosystem stability (Dearden *et al*., 2017; Teem *et al*., 2020) or even agricultural pests (Scott *et al*., 2018). When efforts to reduce the incidence of vector-borne disease (Jones *et al*., 2021) and invasive species (Dearden *et al*., 2017) are stalled, gene drives could potentially offer a highly effective and inexpensive alternative. In other situations, a gene drive approach could be more environmentally friendly or ethical compared with methods involving pesticides or trapping.

For gene drives to be useful, several challenges must be overcome. Some are socio-political in nature and researchers are also faced with a daunting array of technical challenges. For example, resistance alleles formed by the drive itself can halt the spread of the drive. Drive fitness costs, which can be caused by expression of drive components or undesired cleavage of essential genes, could also reduce drive speed and prevent successful outcomes. On the other hand, some types of drives could spread without limit through a species, even if only certain populations should be targeted. With many possible applications of gene drives involving insects, the model organism, *Drosophila melanogaster*, is well placed to be a test bed for gene drive strategies that are designed to overcome these challenges. Indeed, most forms of gene drive thus far have been tested and developed in the fruit fly (Table 9.1), often successfully. Here, we examine each of these drives and their experimental demonstrations in *D. melanogaster*, focusing on mechanisms and lessons learned for developing high-efficiency gene drive systems.

*Email: jchamper@pku.edu.cn
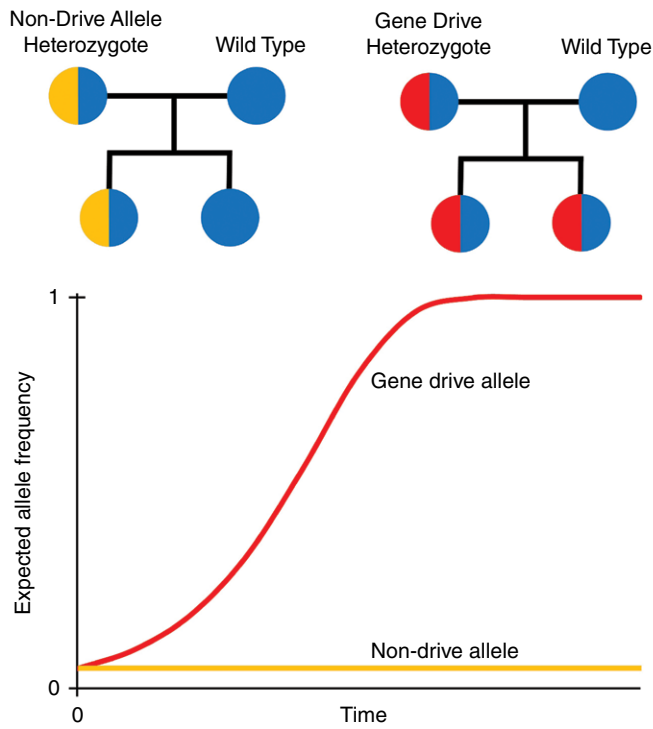
Fig. 9.1. Gene drive inheritance. Non-drive alleles (orange) are inherited by half of the offspring of a heterozygote, remaining at the same frequency in a population on average if they have no fitness effects. A gene drive allele (red), however, can be inherited by all offspring of a drive heterozygote, resulting in a rapid increase in the frequency of the drive allele in the population.

9.2 Engineered Transposon Drives

Transposons are naturally occurring genetic elements that, under the right circumstances, are able to copy themselves into other locations in a genome (see O'Brochta *et al.,* Chapter 1, this volume; Mérel *et al.,* 2020). Since this usually happens in the germline, this will increase their allele frequency in the next generation. While such transposons have been of great use for genetic engineering, they would require alteration to be used as an effective modification gene drive. This is because the natural rate of transposition is very low, meaning that it could not overcome even a small fitness cost of a cargo gene. An early effort in *Drosophila* to engineer transposons for use as a gene drive achieved acceptable efficiency, though success was mixed in different replicates (Carareto *et al.,* 1997). However, further development of transposons as gene drives in other species has stalled. This is perhaps because most organisms have natural resistance to transposons via piRNAs and other mechanisms (Mérel *et al.,* 2020). Furthermore, if a highly efficient transposon-based system were designed, it would need to have some sort of limitation to act as a modification drive. Otherwise, an increase towards unlimited copy number would eventually impose a high fitness cost, leading to population suppression.

9.3 Homing Drives

9.3.1 Basic characteristics

The homing drive is the best-studied type of engineered gene drive and is among the most powerful in terms of its ability to increase quickly in frequency in a population.

Table 9.1. Comparison of major classes of engineered gene drives. Speed and confinement ratings are for the most likely drive parameters (low fitness costs, high cut rates, etc.) and can often vary between drive subtypes. Engineering difficulty refers to the difficulty in generating an efficient form of the drive in both *D. melanogaster* and other relevant species (this can be highly variable depending on species and exact subtype of drive); 'proven' refers to a technical demonstration in *D. melanogaster*; and 'successful' refers to adequate spread in a multigenerational cage experiment.

Drive type	Purpose	Speed	Confinement	Engineering difficulty	Status in flies
Transposon	Modification	Fast	None	High	Proven (Carareto *et al.*, 1997)
Homing	Either	Fast	None	Low	Proven (Champer *et al.*, 2017, 2018, 2019a; S.E. Champer *et al.*, 2020; Gantz and Bier, 2015b; Kandul *et al.*, 2021; Carrami *et al.*, 2018; López Del Amo *et al.*, 2020a; Terradas *et al.*, 2021); and successful (Champer *et al.*, 2020d); proven for suppression (Oberhofer *et al.*, 2018; Yang *et al.*, 2022)
Driving Y X-shredder	Suppression	Fast	None	High	Autosomal proven (Fasulo *et al.*, 2020)
Wolbachia elements	Modification	Medium	Some	Moderate	Proven (Shropshire and Bordenstein, 2019)
Medea	Modification	Medium	Some	High	Successful (Chen *et al.*, 2007)
RNAi underdominance	Modification	Slow	High	High	Successful (Akbari *et al.*, 2013; Reeves *et al.*, 2014)
Chromosomal translocations	Modification	Slow	High	High	Successful (Buchman *et al.*, 2018; Foster *et al.*, 1972)
Species-like incompatibilities	Modification	Slow	High	Moderate	Proven (Maselko *et al.*, 2020)
CRISPR toxin–antidote	Either	Medium	Some	Low	Successful (Champer *et al.*, 2020c; Oberhofer *et al.*, 2019, 2020); theory for suppression (Champer *et al.*, 2020b)
CRISPR underdominance	Either	Slow	High	Low	Theory (Champer *et al.*, 2020a)
Killer–rescue	Modification	Medium	High	Moderate	Successful (Webster *et al.*, 2020)

Inspired by naturally occurring homing endonuclease genes (HEGs), engineered homing drives were first proposed relatively recently (Burt, 2003). They are designed to cleave a wild-type allele in the germline of drive heterozygotes. The DNA break then undergoes homology-directed repair (HDR), which copies the drive allele into the wild-type allele, creating drive homozygous cells in the germline and thus biasing inheritance in favour of the drive allele (Fig. 9.2). The rate that wild-type alleles are converted to drive alleles in heterozygotes is often called the 'drive conversion efficiency' or 'homing efficiency' and is the most important performance parameter for homing drives. Through
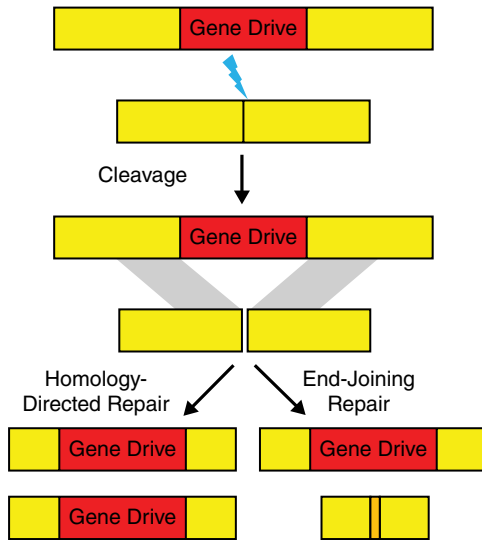
Fig. 9.2. Homing drive mechanism. A homing drive works by cleaving the wild-type allele on its sister chromosome in germline cells, usually with Cas9 and gRNAs. If this break is repaired by homology-directed repair, the wild-type allele will be converted to a drive allele. However, if end-joining repair occurs, the drive is not copied and the target sequence can be mutated, forming a resistance allele that cannot be converted to a drive allele.

this mechanism, a homing drive with at least modest efficiency can rapidly increase in frequency in a population, even if it starts at low initial frequency and carries moderate fitness costs.

In addition to rapidly modifying populations, homing drives can be configured for population suppression though the use of haplosufficient genes, in which one functioning copy is sufficient for an organism to maintain high fitness, but lack of one functioning copy results in a deleterious phenotype. If a haplosufficient but essential gene is targeted by the gene drive without a 'rescue' element that restores gene function, then drive homozygotes will be sterile or nonviable. The drive will still be able to spread through heterozygotes, so this will tend to result in population suppression as a greater fraction of the population becomes sterile or nonviable. However, even in simple models, if drive conversion efficiency is less than 100%, the drive would be predicted

to reach an equilibrium frequency below 100%. At this frequency, a genetic load will be imposed on the population, which refers to the reduction in overall reproductive output caused by the gene drive compared with a similar wild-type population. This genetic load may be sufficient to reduce or eliminate the population, depending on a variety of ecological factors (Dhole *et al.*, 2020). The genetic load will usually be greater if a sex-specific gene is targeted, particularly a female fertility gene. This is because suppression requires that only one sex be made sterile or nonviable, and drive alleles will persist longer and reach a higher equilibrium frequency because they will be removed from the population in only the affected sex.

The first homing drive experiments were conducted in *D. melanogaster* using I-*Sce*I (Chan *et al.*, 2011) and I-*Onu*I (Chan *et al.*, 2013b) nucleases, which have inflexible target sites and would thus be difficult to adapt to natural populations. However, these early experiments were nevertheless successful in detecting drive conversion/homing (copying of the drive to wild-type alleles). Additionally, they found substantial differences in drive conversion efficiency when different regulatory elements were used for nuclease expression, suggesting that a good nuclease promoter is critical for construction of efficient drive systems (Chan *et al.*, 2013a). A follow-up study achieved higher efficiency with transcription activator-like effector nuclease (TALEN) and zinc-finger nuclease (ZFN) (Simoni *et al.*, 2014), which allow for targeting of natural DNA sequences at the cost of reduced genomic stability due to repeated DNA elements.

The CRISPR/Cas9 system avoids genomic instability and allows even greater targeting flexibility using separately expressed guide RNAs (gRNAs) to direct the Cas9 nuclease to its target site. This makes CRISPR nucleases (particularly Cas9) the enzymes of choice for gene drives (see Raban and Akbari, Chapter 8, this volume). The first CRISPR homing drive targeted the X-linked *yellow* gene in *D. melanogaster* and was initially reported to have high efficiency based on

recessive disruption of *yellow* by drive alleles (Gantz and Bier, 2015b). However, actual efficiency was revealed to be lower by another study using a fluorescent marker that showed drive conversion taking place only in the germline, rather than in the early embryo after fertilization (Champer *et al.*, 2017). The yellow phenotype seen in the initial study was caused by the formation of resistance alleles (Fig. 9.3) that disrupted *yellow*. Resistance alleles are target sequence mutations that prevent recognition by the drive's gRNAs, thus preventing drive conversion. They can be formed by end-joining

repair after drive cleavage, which can mutate the target sequence, usually disrupting the target gene's function due to frameshifts or other changes. Such disrupted alleles are often referred to as 'r2' resistance alleles, while less common 'r1' resistance alleles preserve the function of the target gene. Depending on the drive design, these resistance alleles (particularly functional r1 alleles in suppression drives) can cause failure of the drive, because they prevent recognition by the drive's gRNA(s).

Resistance alleles could be formed at three stages: (i) in the germline; (ii) in the
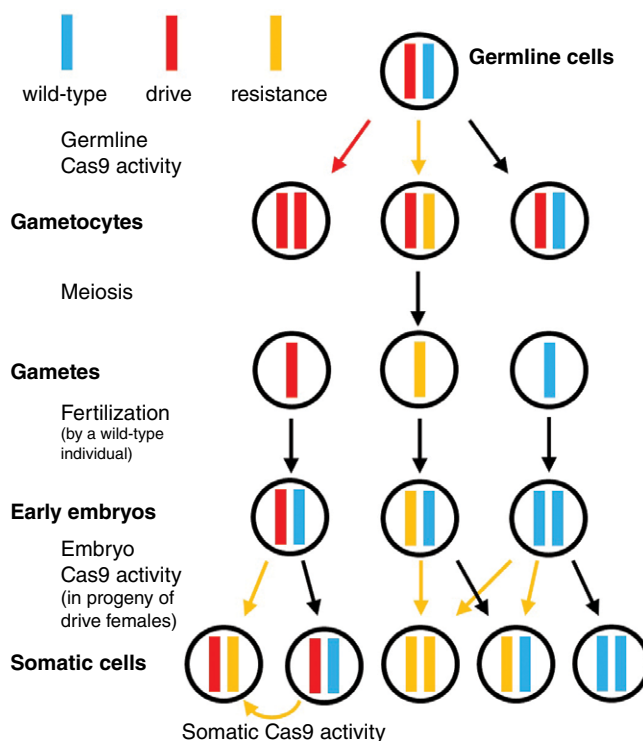
Fig. 9.3. Mechanisms of CRISPR-based homing drives and toxin–antidote drives. In germline cells, nuclease cleavage can result in drive conversion (red arrow) or resistance allele formation (orange arrow). In CRISPR toxin–antidote drives, drive conversion does not take place and conversion of wild-type alleles to disrupted alleles (equivalent to resistance alleles that disrupt the function of their target gene) is desired at this stage. These gametes undergo meiosis and fertilization (in this case by a wild-type individual). However, parentally deposited nuclease (usually maternal) can continue to cleave wild-type alleles, converting them into resistance/disrupted alleles (orange arrows, which is undesired in homing drives and desired at this stage in some CRISPR toxin–antidote drives). The whole organism can have the resulting genotype in some cases, while in other cases nuclease cleavage is mosaic, only occurring in some cells. Later, additional nuclease expression and cleavage can occur in somatic cells, and this can also be mosaic.

early embryo due to maternally deposited Cas9 and gRNA; and (iii) in somatic cells due to 'leaky' expression of Cas9 and subsequent cleavage events (Fig. 9.3). In the germline, resistance alleles could form as an alternative to homology-directed repair in gametocytes. However, some evidence indicates that resistance alleles can also form in pre-gonial germline cells as well, due to early germline expression of Cas9 (Champer *et al.*, 2017, 2018; Carrami *et al.*, 2018). These alleles could potentially be inherited by multiple offspring. Because such resistance allele formation is an alternative to homology-directed repair in the germline, it also has the effect of reducing the drive conversion efficiency, which can have a particularly negative effect on the performance of suppression drives by reducing their genetic load. In the early embryo, drive conversion based on homology-directed repair has not been observed and resistance alleles could form even in cases where the drive allele was not inherited (Champer *et al.*, 2017). This cleavage could occur in the zygote or early embryo, resulting in all cells of the organism possibly inheriting resistance alleles and being unable to perform drive conversion even if they also inherit a drive allele. If Cas9 cleavage is delayed, it can result in mosaic phenotypes where only some cells possess resistance alleles, which does not necessarily prevent drive conversion (Champer *et al.*, 2019a) but could still have negative fitness consequences. A genome-wide association study examining drive performance parameters found that genetic variation had little effect on the rate of drive conversion and germline resistance allele formation, but genetic background substantially impacts the rate of embryo resistance allele formation (Champer *et al.*, 2019b). Somatic expression of Cas9 and subsequent DNA cleavage also could convert wild-type alleles to resistance alleles, though the rate at which it might also promote drive conversion via homology-directed repair has not yet been assessed. Somatic expression was clearly seen when Cas9 was driven by the *vasa* promoter (Gantz and Bier, 2015b; Champer *et al.*, 2018), resulting in drive/wild-type heterozygotes having a similar phenotype to drive/r2 resistance allele heterozygotes. Lower levels of somatic cleavage can result in mosaic phenotypes. Though such somatic resistance may not impact germline drive conversion, it can be highly detrimental to drives with essential target genes, particularly suppression drives, since drive heterozygous individuals may be shifted toward a nonviable or sterile phenotype. Note that Cas9 activity at all these stages also depends on the presence of gRNAs. However, studies thus far have used U6 promoters for universal gRNA expression, potentially allowing cleavage in all cells where Cas9 is also present. Additional restriction of gRNA expression to germline cells could thus potentially reduce resistance allele formation.

9.3.2 Improved versions

With resistance alleles being the primary obstacle for generating successful gene drives, at least for the proven systems in *Drosophila* and *Anopheles*, several efforts have been made to avoid or eliminate them. The first of these used the *nanos* promoter to successfully avoid somatic expression of Cas9, although drive conversion rates and other resistance allele formation rates were not substantially affected (Champer *et al.*, 2018). Using similar drives at different target sites had a large effect on the embryo resistance allele formation rate, ranging from 20% to 100% (Champer *et al.*, 2017, 2018).

The greatest improvement came from using two gRNAs as part of the drive that targeted adjacent sites in the *white* gene (Champer *et al.*, 2018). This increased drive conversion efficiency because there were two opportunities to cut the target gene and induce drive repair, potentially enabling successful drive conversion even after one target site was cut and repaired by end joining. Another study with a suppression-type homing drive targeting a female fertility gene (Fig. 9.4) with four gRNAs (Oberhofer *et al.*, 2018) had substantially lower drive conversion efficiency than similar drives with a single gRNA. In this case, the gRNAs were too far apart for a cut at a single gRNA
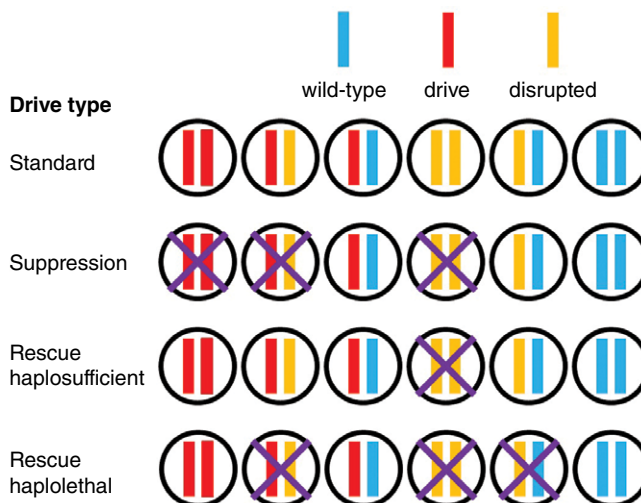
Fig. 9.4. Genotypes of homing and CRISPR toxin–antidote gene drives. In a *standard homing drive*, all genotypes are viable, regardless of which combination of drive, wild-type and disrupted (non-functional resistance) alleles exist. In *suppression drives*, the drive targets a haplosufficient gene without rescue. Thus, organisms that lack wild-type alleles may suffer negative effects (in the most common type of suppression drive, a female fertility gene is targeted, so these individuals are sterile if female). For *rescue drives* (which can be homing rescue drives or CRISPR toxin–antidote drives), a *haplosufficient* target results in only disrupted allele homozygotes being nonviable. This includes drive types such as ClvR and TARE. For rescue drives targeting *haplolethal* genes, any genotype with disrupted alleles is nonviable, even if it contains a drive allele. Note that drive/wild-type individuals with a drive that has somatic cleavage will tend to suffer the effects of a drive/disrupted allele heterozygote. Functional resistance alleles will have the same genotype effects as wild-type alleles (except that they would not be vulnerable to such somatic nuclease expression and cleavage in drive/functional resistance allele heterozygotes).

site to allow effective homology-directed repair, due to lack of immediate homology on one or both sides of the cut site. This is because the DNA adjacent to drive alleles only includes part of the outermost gRNA target sites and none of the inner sites to avoid cleavage of the drive chromosome. This drive also suffered from instability due to repetitive gRNA promoter elements, resulting in recombination and removal of some drive elements at an appreciable rate. This latter issue could likely be solved by use of a tRNA system to express multiple gRNAs from a single promoter, splicing them apart based on recognition of tRNAs between the gRNAs (Port and Bullock, 2016). Using this method, another study examined experimental data from a series of multiple-gRNA drives and developed a performance model for various multiplexed gRNA homing drive configurations (S.E. Champer *et al.*, 2020). In this model, drives were found to have an optimum number of gRNAs, above which drive conversion efficiency decreases. This was because of the difficulty of homology-directed repair in drives that fail to cut at the outer gRNA target sites as described above, but also due to saturation of Cas9 activity, where cleavage rates at individual gRNA target sites are reduced as the number of gRNAs increases with a constant amount of Cas9. Obtaining maximum benefits from multiple gRNAs requires that the target sites be placed as close together as possible without end joining at one site inducing mutations at an adjacent site. Though the benefits to drive conversion efficiency for multiple gRNAs are modest, this technique is still highly useful for reducing the relative rate of functional compared with nonfunctional resistance allele formation. A recent study using four gRNAs to target a female fertility gene successfully

retained high drive conversion efficiency and prevented formation of functional resistance alleles (Yang *et al.*, 2022). However, population suppression was not successful, due to insufficient genetic load caused in part by a moderate fitness cost of the drive.

Though multiplexed gRNAs coupled with conserved sites and suitable Cas9 promoters may be sufficient to develop effective suppression drives, modification drives may be more difficult, due to the need to address non-functional resistance alleles. If an endogenous gene can be targeted to achieve the desired effect, then such alleles may actually contribute to the purpose of the drive, allowing a robust drive even without high drive conversion efficiency (S.E. Champer *et al.*, 2020). If the purpose of the drive is to spread a cargo gene (such as a gene that prevents disease transmission), then resistance alleles should be eliminated, and the best way to do this is still to target an essential gene, giving non-functional resistance alleles a heavy fitness cost (Fig. 9.4). For a modification drive, the drive itself cannot disrupt the essential gene. To overcome this obstacle, the drive can carry a rescue element, a recoded version of the target gene that is immune to cleavage because it no longer is a match for the drive's gRNAs. The first demonstration of such a modification type of homing drive targeted conserved sites in the *RpL35A* gene with two gRNAs to avoid functional resistance alleles (Champer *et al.*, 2020d). *RpL35A* is haplolethal, meaning that individuals require two functioning copies to be viable. Thus, the drive included a recoded version of the 3′ end of the gene downstream of the first gRNA target site, enabling drive alleles to carry a functional copy of *RpL35A*. Non-functional resistance alleles would be inviable at the embryo stage, regardless of whether they were paired with drive or wild-type alleles, resulting in their immediate removal from the population (Fig. 9.4). This drive was highly successful, rapidly spreading through a cage population. Two later studies similarly targeted a haplosufficient but essential viability gene with a single gRNA and a rescue element (Kandul *et al.*, 2021; Terradas *et al.*, 2021). They performed well, but ultimately formed functional

resistance alleles. Comparing these target classes, targeting a haplolethal gene allows for faster removal of non-functional resistance alleles than targeting haplosufficient genes (S.E. Champer *et al.*, 2020). Also, if the drive has a fitness cost, then targeting a haplosufficient gene will tend to result in some non-functional resistance alleles persisting in the long term. On the other hand, an advantage of targeting a haplosufficient gene is that somatic expression (and to a lesser extent embryo resistance) will not have any negative fitness effects, since the rescue element in the drive will prevent target-related fitness effects in drive-carrying individuals. Such expression could result in substantial fitness costs or nonviability in individuals with a drive that targets a haplolethal gene. Though the fruit fly *nanos* promoter used in the haplolethal drive had no detectable somatic expression and modest maternal Cas9 persistence in the embryo, tolerance of somatic and embryo activity could make it easier to construct homing modification drives targeting haplosufficient but essential genes in some less genetically well-characterized species. Even in *Drosophila*, working with haplolethal and generally haplo-insufficient genes is more difficult than with other essential genes. One general issue with homing drives containing rescue elements is that partial homology-directed repair can result in only the rescue element being transferred to the wild-type allele, thus essentially forming a functional resistance allele. Though partial repair of a sufficient large DNA element (enough to cover a normal-sized rescue element) has not been observed, smaller instances have been seen (Champer *et al.*, 2018). It remains unclear how large an issue this may be, though it is certainly possible that a well-designed rescue element could reduce its likelihood to a similar level as payload inactivation by normal errors in homology-directed repair, thus making it a less important consideration (Champer *et al.*, 2020d; S.E. Champer *et al.*, 2020). Of note, the reduced Cas9 expression in the haplolethal homing drive (Champer *et al.*, 2020d) and 4-gRNA homing suppression drive (Yang *et al.*, 2022) appeared to equalize drive conversion efficiency in males

and females, which was previously significantly lower in males when Cas9 expression (as measured indirectly by early embryo resistance allele formation) was higher (Champer *et al.*, 2018, 2019a; S.E. Champer *et al.*, 2020).

### 9.3.3 Variants for drive control and applications

Though only a few years old, CRISPR homing drives have proved surprisingly versatile. Since these are generally rapidly spreading drives, there has been interest in being able to remove a homing drive from a wild population. By directly targeting the homing drive with another 'overwriting' homing drive, one study was able to successfully inactivate the first homing drive (Xu *et al.*, 2020). Some genetic elements would be left in the genome, but any specific desired elements of the first gene drive could be inactivated, and the overwriting gene drive can be designed so as not to spread through natural populations on its own, if desired (though the first drive could likely spread first through a whole population anyway before inactivation (Girardin *et al.*, 2019)). Small molecules have also been used to control gene drives. In one study, a small molecule was provided to activate a modified form of Cas9, permitting drive conversion only when present (López Del Amo *et al.*, 2020b). In another study, a small molecule induced a recombinase that removed itself and the gene drive at a moderate rate per generation (Chae *et al.*, 2020). Though interesting demonstrations, it is less clear how easily these methods could be used for a gene drive that is in the process of spreading through a natural population, since an effective delivery system would be required.

Other drive variants involve rearrangement and/or retargeting of certain drive elements. In allelic drive, the purpose is to increase the frequency of a particular allelic variant of a gene in a population (Guichard *et al.*, 2019). This could simply be a mutation adjacent to a homing drive allele, because DNA around the drive within up to several hundred nucleotides tends to replace the sequence in the cut chromosome (in addition to copying the drive allele), allowing propagation of a desired allele. However, an allelic drive can also work at a distant site if it is composed of a CRISPR construct (not necessarily a CRISPR homing drive or even a gene drive) that cuts a target sequence away from the main element. At the target site, one chromosome has the target sequence that is a match to the gRNA, while the other chromosome with the desired sequence is not a match (acting equivalently to a resistance allele). After cleavage, the target is replaced by the desired sequence if homology-directed repair occurs, the chance of which is increased compared with a homing drive because the repair mechanism only needs to change up to a few nucleotides rather than inserting an entire drive allele. In another similar method, extra gRNAs in a homing drive were used to target and knock out a desired target gene (Kandul *et al.*, 2020). A method called *trans*-complementing separates the gRNA and Cas9 elements at different genomic sites, with the gRNAs inducing cleavage and homing at both sites (López Del Amo *et al.*, 2020a). This arrangement could potentially have fitness advantages against resistance alleles compared with two independent gene drive systems at the cost of somewhat slower spread.

9.4 Shredder Drives

Shredder-based drive systems are designed to create a biased sex ratio in a population, causing suppression by reducing or eliminating either females or males. They work by targeting a sex chromosome (X or Y, alternatively Z or W for some species) in meiotic cells and cleaving it at many sites beyond the ability of DNA repair mechanisms to cope (Fig. 9.5) (see Arien *et al.*, Chapter 10, this volume). This eliminates gametes of one sex but usually results in the same total number of offspring, most or all of which would carry the drive (though effective drive efficiency could be reduced by sperm competition among multiply mated females). To be a gene drive itself, a shredder system must be
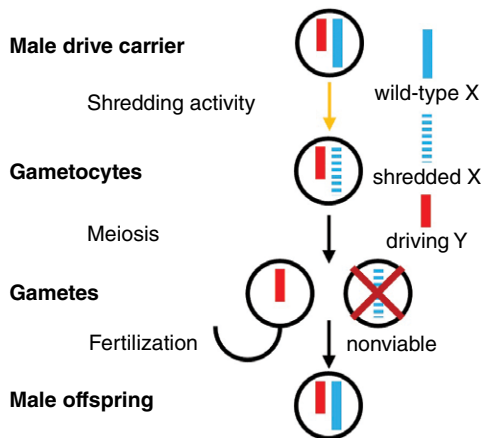
Fig. 9.5. Mechanism of Driving Y/X-shredder gene drives. In the germline of males, the X-shredder gene on the Y chromosome activates, destroying gametes that contain X chromosomes. Since Y-bearing sperm will remain viable, this results in most or all offspring being male.

located on the sex chromosome that is not shredded. This will allow it to be preferentially inherited in addition to biasing the sex ratio. For example, an X-shredder allele located on the Y chromosome is called a Driving Y because it eliminates the X chromosome, thus promoting increased inheritance of the Driving Y chromosome and a male bias among offspring. However, this localization to the Y chromosome represents a difficult aspect of Driving Y design due to the difficulty of generating knock-ins to the Y chromosome and difficulty in expressing genes at a high level from the Y chromosome, which would be necessary for robust shredding activity. Thus, a successful Driving Y has not yet been engineered, and all experiments with X-shredders have used autosomal loci. Such constructs cannot be considered gene drives, because they do not bias their inheritance, but they nonetheless enable study of the X-shredder mechanism. A Driving X chromosome that incorporates a Y-shredder (Prowse *et al*., 2019) would not have this issue, but by creating a female bias, it might actually increase population sizes unless its efficiency was extremely high, since females are usually much more limiting for a species' reproductive capacity than males.

Unlike other engineered gene drive systems, X-shredders were originally developed in *Anopheles* mosquitoes. These were based on the slime mould nuclease I-PpoI, which naturally targets a repeated site on the *Anopheles gambiae* X chromosome and was engineered to have an optimal level of nuclease activity (Galizi *et al*., 2014). A CRISPR-based X-shredder targeting several somewhat closely spaced sites with identical sequences was subsequently trialled in *D. melanogaster* (Fasulo *et al*., 2020). This construct achieved a moderately male-biased sex ratio when Cas9 was driven by the male-specific *βtub85D* promoter from an autosomal locus. Interestingly, *nanos*-Cas9 lines, despite having high germline Cas9 expression, did not show X-shredding activity, likely due to differences in the timing of Cas9 expression between *nanos* and *βtub85D*.

9.5 Toxin–Antidote Gene Drives

Toxin–antidote drives encompass a large array of molecular mechanisms (both natural and engineered) and have been studied for several decades. These drives all contain at least two elements at one or more drive loci: a toxin, which causes death; and an antidote, which prevents death when paired together with the toxin. Thus, these drives work not by increasing their own copy number (as homing drives do), but, instead, by removing wild-type alleles. This results in frequency-dependent kinetics, since a higher frequency of drive alleles means that wild-type alleles are removed at a higher relative rate. On the other hand, the drive will usually have at least a small fitness cost, and many of the toxin–antidote drive mechanisms also result in the removal of some drive alleles. This means that toxin–antidote drives will have introduction frequency thresholds. Below the threshold, the drive will be eliminated from the population. The drive will only begin to increase in frequency if it is present above the threshold. This can be a powerful mechanism to confine the drive to a desired target population (Fig. 9.6), because if migration is low enough, then the
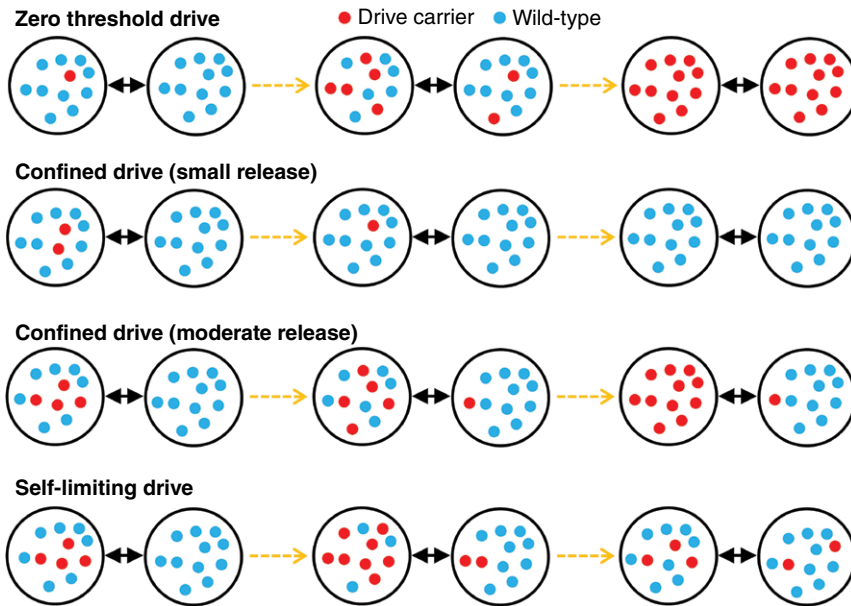
Fig. 9.6. Confinement of gene drives. Several scenarios are shown involving two wild-type (blue) populations connected by migration (double arrows). Gene drive individuals (red) are released into the left population. *Zero-threshold drives* can spread rapidly from even a small initial frequency. Migration will eventually allow them to spread completely through all connected populations. *Confined toxin–antidote drives* will have a release threshold, below which the drive will not be able to spread. Above this threshold, the drive can spread successfully and potentially be prevented from spreading through connected populations if the migration rate is low enough. *Self-limiting gene drives* are designed to spread rapidly at first, but eventually lose their potential to act as a gene drive and disappear from the population (though they can potentially also fix in one or both populations, depending on a variety of factors).

drive will never be present in a non-target population above its critical frequency threshold.

This confinement usually comes at the cost of drive power. Toxin–antidote drives will usually spread more slowly than homing drives or Driving Y systems, require higher release sizes, and, with a couple of exceptions, cannot induce substantial population suppression on their own. On the other hand, toxin–antidote drives tend to be less susceptible to resistance alleles than homing drives, usually because they either do not cut DNA, which potentially induces mutations by end joining, or because they can tolerate or benefit from such end-joining outcomes. As modification drives, they also tend to be more stable than homing-type drives, with a reduced chance of cargo gene inactivation due to mutations that form during replication. This is because toxin–antidote drives are only copied

by normal DNA replication, which has an error rate approximately 1000-fold lower than the homology-directed repair mechanism used by homing drives spreading through a population.

9.5.1 Cytoplasmic incompatibility

Wolbachia are bacteria that infect an incredibly diverse array of insects, including *D. melanogaster*. They are maternally inherited and the key to their success is that they have evolved to spread through populations like a gene drive. They achieve this via cytoplasmic incompatibility, wherein embryos with a *Wolbachia*-infected father are not viable unless they contain maternally inherited *Wolbachia*. Because wild-type females, but not *Wolbachia*-infected females,

suffer from reduced average reproductive success when *Wolbachia*-infected males are present, this enables the *Wolbachia* to spread through a population despite carrying fitness costs. This is useful, since *Wolbachia*-carrying females are often less likely to spread diseases such as dengue (Ross *et al*., 2019). Recently, two genes (*cif*A and *cif*B) that are responsible for this cytoplasmic incompatibility were isolated from *Wolbachia* bacteriophage and used to engineer a synthetic version of *Wolbachia* gene drive in *Drosophila* (Shropshire and Bordenstein, 2019). If an embryo's father had both genes, then the embryo would be inviable unless the mother also had *cif*A (Fig. 9.7). If used as a drive, then this system would have an introduction frequency threshold of 37% in the absence of additional fitness costs.

9.5.2   *Medea*

Originally discovered in red flour beetles, a synthetic maternal effect dominant embryonic arrest (*Medea*) drive was successfully engineered in fruit flies, consisting of a single allele with two elements (Chen *et al*., 2007). The first element is a microRNA (miRNA) toxin targeting *myd88*, a gene transcript that is provided to embryos maternally and is required for their viability. The antidote contains a recoded copy of *myd88* that is immune to the toxin. It is driven by the *bnk* promoter for expression in the zygote, thus rescuing embryos from the toxin if they inherit a *Medea* allele. In this manner, all offspring of *Medea*-carrying females will not be viable unless they inherit a *Medea* allele from either parent (Fig. 9.7). This type of drive has no intrinsic introduction frequency threshold, but any fitness cost will provide a non-zero introduction threshold and prevent drive fixation (though all individuals would still have at least one *Medea* allele). *Medea* was successful in spreading through a population in cage experiments (Chen *et al*., 2007). A follow-up study using two *Medea* drives that targeted different genes found that they had moderate fitness costs (Akbari *et al*., 2014). Though successful

in flies, efforts to bring *Medea* to other species have thus far not been successful, due to the highly specific nature of the target gene and rescue element promoter.

9.5.3   RNAi underdominance drives

Underdominance refers to heterozygote disadvantage, in which the fitness of heterozygotes is lower than the fitness of either homozygote. A gene drive with this characteristic would have an introduction frequency threshold greater than zero even if it did not induce any fitness costs in drive homozygotes. Thus, these drives tend to be very stringently confined compared with other toxin–antidote systems.

The first engineered RNAi-based underdominance drive in *Drosophila* targeted the haplo-insufficient gene *RpL14* with RNAi while providing a rescue element consisting of a recoded *RpL14* immune to the RNAi (Reeves *et al*., 2014). Since the RNAi is efficient at knocking down wild-type *RpL14* expression, drive heterozygotes would thus suffer substantial fitness costs because they would only carry a single effective *RpL14* copy (their recoded form). This would eliminate both drive and wild-type alleles from the population equally in these heterozygotes (Fig. 9.7). With no unintended fitness costs, such a system would have a high introduction frequency threshold of 50%. In the experimental system, drive homozygotes did have a moderate fitness cost, but drive heterozygotes had a far higher cost, allowing the drive to spread successfully through cage populations when released above 61% frequency (the drive's introduction threshold, increased above 50% due to the homozygote fitness costs).

Other forms of RNAi-based drive designs were initially proposed in a modelling study (Davis *et al*., 2001). These involve two different drive alleles that each target a separate essential (usually haplosufficient) gene. However, each type of drive allele provides rescue for the gene targeted by the other drive allele (Fig. 9.7). Thus, individuals are only viable if they have at least one

Cytoplasmic incompatibility drive

	♂ C	+
♀ +	none	+

Medea drive ♂

	+	+
♀ M	M+	M+
+	++	++

♂

	M	+
♀ M	MM	M+
+	M+	++

RNAi underdominance drive

| Rescue | RNAi | → Target gene |

Genotype
DD
D+
++

Reciprocal chromosomal translocations drive

Chromosome 1 Chromosome 2

+ A   + B

	BB	B+	++
AA	AA/BB	AA/B+	AA/++
A+	A+/BB	A+/B+	A+/++
++	++/BB	++/B+	++/++

Two-allele underdominance drives

| A | Rescue 1 | Toxin 2 |
| B | Rescue 2 | Toxin 1 |

1-Locus

AB	AA	A+	BB	B+	++

2-Locus

	BB	B+	++
AA	AA/BB	AA/B+	AA/++
A+	A+/BB	A+/B+	A+/++
++	++/BB	++/B+	++/++
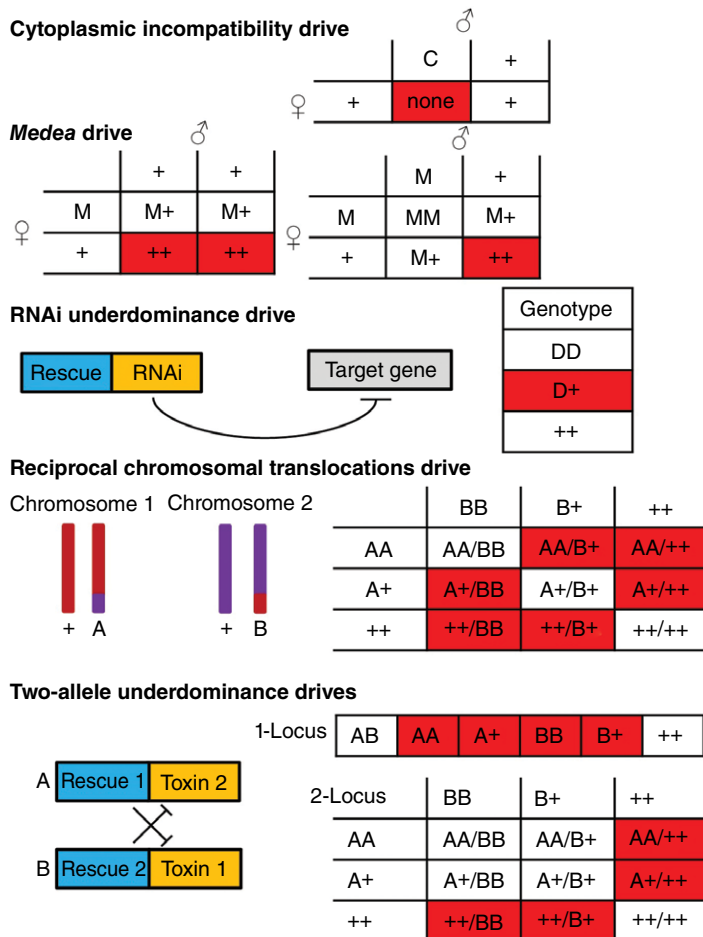
Fig. 9.7. Mechanisms of toxin–antidote drives. *Cytoplasmic incompatibility drive* using *Wolbachia* phage genes functions by preventing wild-type (+) females from having offspring with drive-carrying (C) males. Drive-carrying females will have viable progeny regardless of whether their mate has the drive or not, thus resulting in greater reproductive success for drive carriers if the drive frequency is above the introduction threshold frequency of 37%. *Medea* (maternal effect dominant embryonic arrest) works by killing all offspring that receive a maternal toxin from the drive allele (M) unless they also inherit the drive allele from either parent. Crosses that can have nonviable offspring are shown. *Medea* has a zero-introduction threshold, but this increases with any fitness costs. *Single-allele RNAi underdominance drives* (D) (and *species-specific incompatibility* alleles, which have a different mechanism but share the same genotype viability table) will be fully viable when in homozygotes, but will cause large fitness costs or nonviability in heterozygotes. *Reciprocal chromosomal translocations* result in most genotypes being nonviable, because they will have incorrect copy numbers of two large chromosome segments. Since effects are similar for drive and wild-type alleles, this drive has an introduction threshold frequency of 50%. *Two-allele underdominance drives* involve alleles that each provide rescue for the other's toxin (see Edgington and Alphey, Chapter 12, this volume). If arranged at the same locus, then most drive genotypes are nonviable, resulting in an introduction threshold frequency of 67%. If at separate loci, more drive genotypes are viable, resulting in an introduction threshold frequency of 27%. Both of these decrease somewhat if CRISPR underdominance toxin–antidote alleles are used instead of RNAi-based toxins.

copy of each type of drive allele. If these alleles must share the same genomic locus, only heterozygotes for both drive alleles will be viable and the introduction frequency threshold will thus be 67%, given no additional fitness costs from the drive. This is because the only crosses involving drive individuals that produce viable progeny are those between heterozygotes for each type of drive, which results in only half as many viable offspring as crosses between wild-type individuals. If each drive allele type has its own unlinked locus, then many drive-containing genotypes will be viable because each locus only needs one copy of their drive. This means that more wild-type alleles than drive alleles will be removed in inviable genotypes. This substantially reduces the introduction frequency threshold to 27% (for an introduction of double drive homozygotes) in the absence of additional fitness costs. Both these forms were demonstrated using rearranged *Medea* allele elements (Akbari *et al.*, 2013).

9.5.4 Other underdominance drives

The first engineered gene drive systems involved chromosomal translocations (Curtis, 1968; Whitten, 1971), where chromosome segments could be broken and fused. In *Drosophila*, compound chromosomes were formed by fusing two copies of the left arm together and separately fusing two copies of the right arm of a chromosome together (Foster *et al.*, 1972). Individuals homozygous for such mutations could only have viable offspring with other homozygotes; even then, three-fourths of their offspring would be nonviable. This would result in an introduction frequency threshold of 80%, though the actual experimental demonstration resulted in additional fitness costs that increased this to approximately 90%.

Another form of this type of drive is reciprocal chromosomal translocations, where two segments of DNA are removed from separate chromosomes and then switched with each other. Because these segments contain many essential genes, individuals will only be viable if they have all wild-type chromosomes, all rearranged chromosomes, or one of each type of both rearranged and wild-type chromosomes (Fig. 9.7). Since all other genotypes will be inviable, this results in an introduction frequency threshold of 50% in the absence of additional fitness costs. Early chromosomal translocations in mosquitoes were generated with radiation, resulting in high fitness costs (Lorimer *et al.*, 1972). A recent study in flies used CRISPR methods to generate the translocations, reducing the additional fitness costs (Buchman *et al.*, 2018).

Another drive mechanism with a 50% introduction frequency threshold is species-like incompatibility. In this method, the drive-carrying individuals do not produce any viable offspring with wild-type individuals (Fig. 9.7). There are many possible mechanisms to achieve this, and a recent demonstration used two components (Maselko *et al.*, 2020). The first was an allele based on dCas9 that binds to and creates lethal overexpression of a target gene and the second was a recessive suppresser of this lethal element consisting of mutations at the target gene promoter that block dCas9 binding. Thus, heterozygotes would carry only one copy of the suppresser and not be viable because overexpression would still take place at copy of the one target gene.

9.5.5 CRISPR toxin–antidote drives

The previously described toxin–antidote approaches remain as viable methods for gene drive, but all can be difficult to engineer, making them potentially less portable between fruit flies and other organisms of interest such as mosquitoes. To address this, CRISPR toxin–antidote systems have been developed that function based on simple principles. In these systems, the toxin is a CRISPR nuclease (Cas9) targeting an essential gene and the antidote is a recoded copy of the gene that is immune to the CRISPR nuclease. The drive allele thus converts wild-type alleles to disrupted alleles (Fig. 9.3), removing them from the population (Fig. 9.4) in a frequency-dependent manner. Unlike homing drives, wild-type alleles are never

converted into drive alleles. One important advantage of these systems over homing drives is that functional resistance alleles can be more easily avoided with multiplexed gRNAs without loss of drive efficiency. This is because both homology-directed repair and end joining would still convert the target gene to a disrupted allele. Thus, CRISPR toxin–antidote systems should, in practice, be able to avoid forming functional resistance alleles, like other toxin–antidote systems.

The first experimental demonstrations of CRISPR toxin–antidote systems targeted essential but haplosufficient genes using Cas9 driven by the *nanos* promoter and multiple gRNAs in *Drosophila* (Oberhofer *et al.*, 2019; Champer *et al.*, 2020c). In these two systems, drive alleles would always be viable, since they had a recoded copy of the haplosufficient target gene. However, individuals inheriting two disrupted alleles for the target would not be viable if no drive alleles were present. Because end-joining repair of the target was desired, maternally deposited Cas9 and gRNAs (Fig. 9.2) actually contributed to the efficiency of these drives, since they allowed wild-type to be converted to disrupted alleles more quickly, resulting in immediate inviability in most progeny of drive females that failed to inherit the drive (Fig. 9.4). This drive mechanism has no introduction frequency threshold in the absence of fitness costs, but any fitness cost will result in a non-zero threshold. Such fitness costs would also prevent the drive from reaching fixation, though at long-term equilibrium all individuals would still have at least one drive allele. Both of these CRISPR toxin–antidote drive examples were able to spread rapidly through cage populations (Oberhofer *et al.*, 2019; Champer *et al.*, 2020c). Their main difference lies in the localization of the drive allele. Toxin–antidote recessive embryo (TARE) drive is inserted directly into the target gene ('same-site') and directs its gRNAs to cut the gene downstream of the drive insertion site (Champer *et al.*, 2020c). Cleave-and-rescue (ClvR) is located at a 'distant-site' from the target gene, containing a complete recoded copy of this gene and directing its gRNAs anywhere in the target. Each method has its advantages.

Same-site drives have a higher likelihood of successful rescue because the recoded rescue element is at its native genomic site, with its natural regulatory elements already in place (thus also likely requiring a smaller transgenic package, since the promoter need not be included in the transgenic DNA to be inserted), but distant-site drives can be placed more flexibly in the genome and can target smaller genes because they do not need to cleave downstream of a drive insertion site. A follow-up study demonstrated effective replacement of one ClvR drive in a population with a second drive (Oberhofer *et al.*, 2020).

There are many more useful CRISPR toxin–antidote drive types that can presumably be readily engineered with already available and demonstrated genetic elements. For example, an underdominance drive with an intrinsic introduction threshold frequency (regardless of fitness costs) could also be developed by using two types of reciprocally targeting CRISPR toxin–antidote drive alleles (Champer *et al.*, 2020a). Like the RNAi designs, these could be at the same or different genetic loci to vary the introduction threshold frequency (Fig. 9.7). A confined suppression drive could even be engineered by targeting and recoding a haplolethal gene (instead of an essential but haplosufficient gene) and either placing the drive in an essential but haplosufficient female fertility gene or disrupting such a gene with additional gRNAs (Champer *et al.*, 2020a,b). Depending on the Cas9 promoter, such a drive could also have a variable introduction threshold frequency (Champer *et al.*, 2020a,b).

9.5.6   Tethered drives

While not an independent design itself, 'tethering' a drive refers to using a toxin–antidote system to provide an essential component of a more powerful drive form, usually a homing drive (Dhole *et al.*, 2019). In this manner, the homing drive can only perform drive conversion in the presence of the toxin–antidote system, which can provide a nuclease such as Cas9. This allows the system to be confined to a target population

like the toxin–antidote system but with the power of a homing drive to facilitate population suppression or modification with costly cargo genes. Since the type of underdominance system used to tether a drive is flexible, CRISPR toxin–antidote systems are ideal candidates, particularly if they could share a Cas9 element with the homing element. This was recently demonstrated when a TARE drive was used to propel a population suppression and a population modification homing drive, itself lacking Cas9, to high frequency in population cages over several generations (Metzloff *et al.*, 2022).

9.6 Self-limiting Gene Drives

A self-limiting gene drive (Alphey *et al.*, 2020; Gould *et al.*, 2008) can still promote biased inheritance, but it has some inherent mechanism that will, ideally, doom it to elimination from the population in a reasonable time frame (Fig. 9.6). The details of how this is accomplished depend on the specific drive mechanism. Overall, though, this provides a potential mechanism to keep the gene drive present for only a limited time, potentially easing regulatory constraints on the deployment of such drives and enabling their confinement to a target area. However, if released at a high enough frequency, some types of self-limiting drives may still be powerful enough to spread completely through both a target area and a non-target area if migration between them is high enough. Thus, like with threshold-based toxin–antidote systems, effective confinement of self-limiting drives depends on the ecology and migration patterns of individuals in the intended release areas.

9.6.1 Killer–rescue drives

The killer–rescue mechanism involves two unlinked genetic elements. The 'killer' element induces death with even a single copy, while the 'rescue' element prevents killer-induced death (Fig. 9.8). By releasing
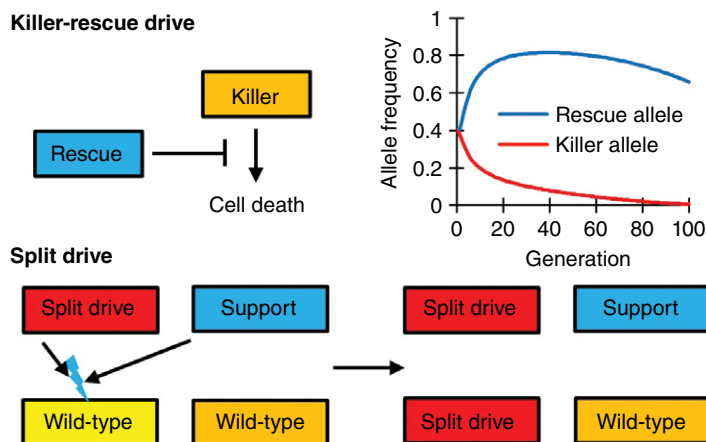
Fig. 9.8. Self-limiting drive mechanisms. A *killer–rescue drive* contains two alleles at separate genomic sites. All individuals with a killer allele will be nonviable unless a rescue allele is also present. The killer allele thus rapidly declines in frequency. However, the rescue allele (the drive element) will initially increase in frequency because wild-type alleles paired with the killer allele are removed from the population. When the killer allele frequency is low, fitness costs of the rescue allele will result in it declining in frequency and eventually being eliminated. *Split drives* can bias their own inheritance, but only when they are together with a supporting element that cannot bias its inheritance. This tends to result in strong drive until the supporting element eventually is eliminated due to fitness costs, at which time the split drive starts declining in frequency (if it has not reached fixation).

constructs with both of these elements, the killer element will over time eliminate itself from the population while propelling the rescue element to high frequency. However, since the rescue element should have at least a small fitness cost, it will not reach fixation, because its selective advantage will be reduced as the killer element declines in frequency. Once the killer element eliminates itself, the rescue element will slowly decline in frequency due to its lower fitness than remaining wild-type alleles. Thus far, there has been only a single experimental demonstration of killer–rescue drives. In *D. melanogaster*, the killer element was composed of a GAL4 gene driven by a promoter that includes an upstream activation sequence (UAS) element (Webster *et al*., 2020). Since GAL4 binds to the UAS element, this element could induce runaway transcription of itself, leading to death. A rescue element used UAS elements to drive GAL80, which could bind the GAL4 and prevent runaway transcription. In multigenerational cage studies, this system behaved as expected, reaching high frequency but not reaching fixation.

9.6.2   Split drives

If a gene drive requires multiple genetic elements for biased inheritance, it can be turned into a split drive system by moving some, but not all, of those elements to a separate genomic site. These elements are not themselves inherited in a biased fashion but instead 'support' the driving element, which can only bias its own inheritance if at least one supporting element allele is present (Fig. 9.8). If the supporting element has a fitness cost, this provides a limit on how long effective drive activity can last, potentially providing temporal and spatial confinement of the drive. In its most common form, the supporting element consists of a Cas9 gene, while the driving element contains the gRNAs and other elements needed for biased inheritance. Split drives have found much use in the laboratory, since they would effectively prevent spread of the drive in the event of an accidental release. In the field, they can be used

for population modification, but they may struggle with suppression due to reduced maximum genetic load when all drive alleles are not always able to provide homing. Additionally, the outcome of a split drive can be highly sensitive to the size of the release if the supporting/Cas9 element has small fitness costs. This reduces their ability to be confined to a target population compared with standard toxin–antidote systems, in which higher release sizes are unlikely to increase their capacity to invade connected populations. While it seems likely that a lone Cas9 element would indeed have small fitness costs (see below), this issue can potentially be mitigated by placing the Cas9 inside a gene where disruption induces moderate fitness costs.

Originally developed in yeast (DiCarlo *et al*., 2015), split drives were first demonstrated in flies using a split homing element targeting the *yellow* gene (Champer *et al*., 2019a). The split drive had higher drive conversion than a standard *nanos*-Cas9 homing drive at the same site, likely due to higher Cas9 expression or a smaller drive element. However, this same higher Cas9 expression from its autosomal locus also resulted in higher resistance allele formation in the early embryo. 'Shadow drive' was also observed, in which germline drive conversion could still take place at a low rate when powered by maternally deposited Cas9. Since then, split drive systems have commonly been used as a proxy for standard homing drives and CRISPR toxin–antidote gene drives in most fruit fly studies. One study focused on applications of split CRISPR toxin–antidote ClvR elements outside the laboratory, noting that it enabled the drive element to have greater confinement than a standard ClvR system (Oberhofer *et al*., 2021). Indeed, such a split ClvR system would be more confinable than a split homing drive system, not just because the drive element is intrinsically more confined, but also because supporting Cas9 elements would be removed by the drive mechanism even if they had no intrinsic fitness costs. To increase the power of any split drive system, daisy elements can be used. A daisy drive consists of several elements, where each element can

act as a supporting element for drive of the next, with only the first element lacking the ability to bias its inheritance when combined with the correct supporting element (Noble *et al*., 2019). Although using such a design in lieu of split drives allows for smaller releases and potentially enough power for population suppression, it comes at the cost of reduced ease and certainty of drive confinement.

9.7 Measurement of Gene Drive Fitness

In addition to providing a test bed for gene drive systems, fruit flies have been used in a variety of other contexts that support gene drive development. Perhaps the most important is characterization of genes, their promoters, and tools for use of CRISPR nucleases and their gRNAs. Such work often makes targets and individual genetic elements available for use in different types of gene drives. More directly, *Drosophila* have been used to develop methods for assessing the fitness of gene drives. This is a critical parameter in most types of gene drives but particularly in confined drives, due to its effect on their introduction threshold, as well as in suppression gene drives, where lower fitness can have a drastic effect on their performance in spatially explicit environments (North *et al*., 2020; Champer *et al*., 2021).

Some fitness components can be adequately measured by small-scale experiments, but to obtain more realistic fitness estimates, multigenerational population cages should be used to allow fitness effects on as many life history characteristics as possible in a laboratory setting. There are multiple ways to analyse such experiments to obtain fitness values. For example, a recent study analysed the population dynamics of an allele containing a fluorescent protein that disrupted the X-linked *yellow* gene, tracking the frequency of all genotypes over several generations (Liu *et al*., 2019). To prevent any stochastic fluctuation in any individual generation from disrupting the model fit, a maximum likelihood method was used

to analyse each generational transition independently to find the best-fitting model. The technique was quite powerful, detecting strong effects on male mating success with wild-type females but not yellow phenotype females, as expected when the *yellow* gene is disrupted. There was likely also a small negative impact on the viability of all individuals. This technique was later used to determine that a split homing drive (Champer *et al*., 2020d) had little to no fitness impact and that a TARE drive (Metzloff *et al*., 2022) and homing suppression drive (Yang *et al*., 2022) had small or moderate fitness costs, respectively. However, changes in allele frequencies and targeting of native genes can make it difficult to understand fitness effects, due to multiple possible sources of fitness costs. A separate study thus attempted to determine the fitness of non-driving CRISPR elements (Langmüller *et al*., 2021), which could potentially represent 'baseline' fitness costs of gene drives in *D. melanogaster* with similar components. This study revealed that direct fitness costs, if any, were small, but that off-target Cas9 cleavage could impose a moderate fitness cost. This fitness cost was eliminated by use of a 'high fidelity' Cas9 nuclease designed to reduce off-target cutting. Other recent methods of measuring parameters in *Drosophila* have used a variety of approaches. Small fitness costs were found in split ClvR elements using a least-square fit approach (Oberhofer *et al*., 2021), and small to moderate fitness costs were found in split homing drives using a likelihood-based Markov chain Monte Carlo method (Terradas *et al*., 2021).

9.8 Comparisons with Other Organisms

Although most engineered gene drives so far have been in *D. melanogaster*, most applications of such systems will be in other organisms, particularly mosquitoes. Therefore, it is important to consider how closely characteristics of gene drives in *D. melanogaster* relate to those in other organisms. While it is likely that many aspects involving the fundamental

mechanism of most gene drives would be the same across species, gene drives have only been well studied in *Anopheles* mosquitoes, and these have been limited to homing and X-shredder drives. It remains unclear how closely particular quantitative characteristics might be shared among species in a variety of matters such as loss of efficiency from gRNA spacing or magnitude of parental nuclease deposition into embryos.

The spotted wing drosophila (*Drosophila suzukii*) is a widespread invasive species and agricultural pest due to its ability to lay eggs in fresh fruit. It is closely related to *D. melanogaster* and so individual genetic elements can potentially have highly similar performance between these species. Indeed, homologues of some *D. melanogaster* genes have already been shown to perform similarly (Ahmed *et al.*, 2019). Promoter sequences from *D. melanogaster* for Cas9 and gRNAs can even be used in *D. suzukii* (Li and Scott, 2016).

The *vasa* and *nanos* promoters have been used in *D. melanogaster* and *Anopheles* species for expression of Cas9 (see Nolan and Hammond, Chapter 3, this volume), while U6 promoters have been used in both species for gRNA expression. *Vasa* appears to function similarly in these species, with strong germline expression, but also moderate to high somatic expression and maternal deposition into embryos (Gantz *et al.*, 2015; Hammond *et al.*, 2015; Champer *et al.*, 2018). U6 promoters also appear to yield strong ubiquitous expression for gRNAs in both *Drosophila* and *Anopheles*. However, the *nanos* promoter, while giving strong germline expression in both species, exhibits important differences. In *D. melanogaster*, it has strong maternal deposition but no detectable somatic expression (Champer *et al.*, 2017, 2018); in *Anopheles*, maternal deposition is minimal while somatic expression appears to be moderate (Carballal-Lejarazú *et al.*, 2020; Hammond *et al.*, 2021). This is quite important, because somatic expression, and to a lesser extent maternal deposition, can substantially reduce the efficiency of homing suppression drives. More generally, there have been no observed instances of paternal deposition of nucleases

in *Drosophila*, but in *Anopheles* the first X-shredders based on the I-PpoI nuclease had sufficient paternal deposition to shred all embryonic X-chromosomes (Windbichler *et al.*, 2008). This required modification of I-PpoI to sufficiently destabilize it and avoid substantial offspring nonviability (Galizi *et al.*, 2014). It is not yet clear if paternal deposition is important for CRISPR applications in *Anopheles*. Some studies have reported this phenomenon (Galizi *et al.*, 2016; Kyrou *et al.*, 2018; Hammond *et al.*, 2021), but this could potentially also be explained by somatic Cas9 expression. One general difference between *D. melanogaster* and *Anopheles* drives is the higher efficiency observed in the *Anopheles* drives, even when using genetic elements that are homologues in each species. This was seen in autosomal Cas9-based X-shredders (Galizi *et al.*, 2016; Fasulo *et al.*, 2020), but it is most dramatic in homing drives, where *Anopheles* drives rarely fall below 90% drive conversion efficiency (Gantz *et al.*, 2015; Kyrou *et al.*, 2018; Adolfi *et al.*, 2020; Carballal-Lejarazú *et al.*, 2020; Hammond *et al.*, 2021, 2015), while *Drosophila* drives are usually below 80% and average closer to perhaps 60% (Champer *et al.*, 2017, 2018, 2019a, 2020b,d; Carrami *et al.*, 2018; Chae *et al.*, 2020; S.E. Champer *et al.*, 2020; López Del Amo *et al.*, 2020b). Indeed, in an *Anopheles* system, drive conversion was high enough to induce successful population suppression (this drive had a single gRNA, but formation of functional resistance alleles was avoided by targeting a conserved site where all detected mutations disrupted an essential intron–exon junction) (Kyrou *et al.*, 2018).

Gene drives are much less well characterized in other species, with only a few examples of homing drives. In *Aedes aegypti*, for example, U6 promoters combined with germline Cas9 promoters did not appear to be able to achieve the high cut rates and drive conversion efficiency found in *Drosophila* and *Anopheles* (Li *et al.*, 2020; Verkuijl *et al.*, 2020). Drive conversion efficiency in mice was also very low (Grunwald *et al.*, 2019), and drive conversion did not take place at all when Cas9 was driven by the *vasa* promoter (Grunwald *et al.*, 2019). In yeast,

however, not only was drive conversion very high, but also resistance alleles did not form at appreciable rates (DiCarlo *et al.*, 2015; Shapiro *et al.*, 2018; Yan and Finnigan, 2019). Thus, while gene drive mechanisms appear to be conserved across species, there can be substantial performance differences even between similar designs.

9.9 Conclusions

In summary, the model organism *Drosophila melanogaster* remains an excellent system to develop and test various gene drive systems. The knowledge of mechanisms and performance characteristics gained from such experiments, when incorporated into a realistic computational modelling framework (Huang *et al.*, 2011; Eckhoff *et al.*, 2017; Girardin *et al.*, 2019; North *et al.*, 2019, 2020; Champer *et al.*, 2020e, 2021; S.E. Champer *et al.*, 2021; Faber *et al.*, 2021), allows prediction of the outcome of a gene drive release.

This can inform the design and development of gene drives in disease vectors, invasive organisms and other species of interest, allowing greater focus on the most promising strategies. Gene drive research is still in its infancy and such research in the fruit fly is far from finished. There will be more testing of novel forms of gene drive to determine the characteristics and pitfalls. *D. melanogaster* is also an excellent organism for optimizing gene drive strategies by simultaneously testing multiple variants, allowing it to contribute to gene drive research even when the drives themselves are already established in other organisms of interest, eventually allowing deployment of more refined and successful drive systems.

Acknowledgements

References

Adolfi, A., Gantz, V.M., Jasinskiene, N., Lee, H.F., Hwang, K. *et al.* (2020) Efficient population modification gene-drive rescue system in the malaria mosquito *Anopheles stephensi*. *Nature Communications* 11, 1–13.

Ahmed, H.M.M., Hildebrand, L. and Wimmer, E.A. (2019) Improvement and use of CRISPR/Cas9 to engineer a sperm-marking strain for the invasive fruit pest *Drosophila suzukii*. *BMC Biotechnology* 19, 1–14.

Akbari, O.S., Matzen, K.D., Marshall, J.M., Huang, H., Ward, C.M. and Hay, B.A. (2013) A synthetic gene drive system for local, reversible modification and suppression of insect populations. *Current Biology* 23, 671–677.

Akbari, O.S., Chen, C.H., Marshall, J.M., Huang, H., Antoshechkin, I. and Hay, B.A. (2014) Novel synthetic *Medea* selfish genetic elements drive population replacement in *Drosophila*; a theoretical exploration of *Medea*-dependent population suppression. *ACS Synthetic Biology* 3, 915–928.

Alphey, L.S. Crisanti, A., Randazzo, F. (Fil) and Akbari, O.S. (2020) Opinion: Standardizing the definition of gene drive. *Proceedings of the National Academy of Sciences USA* 117, 30864–30867.

Buchman, A.B., Ivy, T., Marshall, J.M., Akbari, O.S. and Hay, B.A. (2018) Engineered reciprocal chromosome translocations drive high threshold, reversible population replacement in *Drosophila*. *ACS Synthetic Biology* 7, 1359–1370.

Bull, J.J. (2015) Evolutionary decay and the prospects for long-term disease intervention using engineered insect vectors. *Evolution, Medicine, and Public Health* 2015, 152–166.

Burt, A. (2003) Site-specific selfish genes as tools for the control and genetic engineering of natural populations. *Proceedings of the Royal Society B: Biological Sciences* 270, 921–928.

Burt, A. and Crisanti, A. (2018) Gene drive: evolved and synthetic. *ACS Chemical Biology* 13, 343–346.

Carareto, C.M.A., Kim, W., Wojciechowski, M.F., O'Grady, P., Prokchorova, A.V. *et al.* (1997) Testing transposable elements as genetic drive mechanisms using *Drosophila* P element constructs as a model system. *Genetica* 101, 13–33.

Carballar-Lejarazú, R. and James, A.A. (2017) Population modification of Anopheline species to control malaria transmission. *Pathogens and Global Health* 111, 424–435.

Carballar-Lejarazú, R., Ogaugwu, C., Tushar, T., Kelsey, A., Pham, T.B. *et al.* (2020) Next-generation gene drive for population modification of the malaria vector mosquito, *Anopheles gambiae*. *Proceedings of the National Academy of Sciences USA* 117, 22805–22814.

Carrami, E.M., Eckermann, K.N., Ahmed, H.M.M., Sánchez Castellanos, H.M., Dippel, S. *et al.* (2018) Consequences of resistance evolution in a Cas9-based sex-conversion suppression gene drive for insect pest management. *Proceedings of the National Academy of Sciences USA* 115, 6189–6194. https://www.pnas.org/doi/10.1073/pnas.2121101118

Chae, D., Lee, J., Lee, N., Park, K., Moon, S.J. and Kim, H.H. (2020) Chemical controllable gene drive in *Drosophila*. *ACS Synthetic Biology* 9, 2362–2377.

Champer, J. Buchman, A. and Akbari, O.S. (2016) Cheating evolution: engineering gene drives to manipulate the fate of wild populations. *Nature Review Genetics* 17, 146–159.

Champer, J., Reeves, R., Oh, S.Y., Liu, C., Liu, J. *et al.* (2017) Novel CRISPR/Cas9 gene drive constructs reveal insights into mechanisms of resistance allele formation and drive efficiency in genetically diverse populations. *PLoS Genetics* 13, e1006796.

Champer, J., Liu, J., Oh, S.Y., Reeves, R., Luthra, A. *et al.* (2018) Reducing resistance allele formation in CRISPR gene drive. *Proceedings of the National Academy of Sciences USA* 115, 5522–5527.

Champer, J., Chung, J., Lee, Y.L., Liu, C., Yang, E. *et al.* (2019a) Molecular safeguarding of CRISPR gene drive experiments. *eLife* 8, e41439.

Champer, J., Wen, Z., Luthra, A., Reeves, R., Chung, J. *et al.* (2019b) CRISPR gene drive efficiency and resistance rate is highly heritable with no common genetic loci of large effect. *Genetics* 212, 334–341.

Champer, J., Champer, S.E., Kim, I.K., Clark, A.G. and Messer, P.W. (2020a) Design and analysis of CRISPR-based underdominance toxin-antidote gene drives. *Evolutionary Applications* 14, 1052–1069.

Champer, J., Kim, I.K., Champer, S.E., Clark, A.G. and Messer, P.W. (2020b) Performance analysis of novel toxin-antidote CRISPR gene drive systems. *BMC Biology* 18, 27.

Champer, J., Lee, E., Yang, E., Liu, C., Clark, A.G. and Messer, P.W. (2020c) A toxin-antidote CRISPR gene drive system for regional population modification. *Nature Communications* 11, 1082.

Champer, J., Yang, E., Lee, E., Liu, J., Clark, A.G. and Messer, P.W. (2020d) A CRISPR homing gene drive targeting a haplolethal gene removes resistance alleles and successfully spreads through a cage population. *Proceedings of the National Academy of Sciences USA* 117, 24377–24383.

Champer, J., Zhao, J., Champer, S.E., Liu, J. and Messer, P.W. (2020e) Population dynamics of underdominance gene drive systems in continuous space. *ACS Synthetic Biology* 9, 779–792.

Champer, J., Kim, I.K., Champer, S.E., Clark, A.G. and Messer, P.W. (2021) Suppression gene drive in continuous space can result in unstable persistence of both drive and wild-type alleles. *Molecular Ecology* 30, 1086–1101.

Champer, S.E., Oh, S.Y., Liu, C., Wen, Z., Clark, A.G. *et al.* (2020) Computational and experimental performance of CRISPR homing gene drive strategies with multiplexed gRNAs. *Science Advances* 6, eaaz0525.

Champer, S.E., Oakes, N., Sharma, R., García-Díaz, P., Champer, J. and Messer, P.W. (2021) Modeling CRISPR gene drives for suppression of invasive rodents. *PLoS Computational Biology* 17, e1009660.

Chan, Y.S., Naujoks, D.A., Huen, D.S. and Russell, S. (2011) Insect population control by homing endonuclease-based gene drive: an evaluation in *Drosophila melanogaster*. *Genetics* 188, 33–44.

Chan, Y.S., Huen, D.S., Glauert, R., Whiteway, E. and Russell, S. (2013a) Optimising homing endonuclease gene drive performance in a semi-refractory species: the *Drosophila melanogaster* experience. *PLoS ONE* 8, e54130.

Chan, Y.S., Takeuchi, R., Jarjour, J., Huen, D.S., Stoddard, B.L. and Russell, S. (2013b) The design and *in vivo* evaluation of engineered I-OnuI-based enzymes for HEG gene drive. *PLoS ONE* 8, e74254.

Chen, C.H., Huang, H., Ward, C.M., Su, J.T., Schaeffer, L.V., Guo, M. and Hay, B.A. (2007) A synthetic maternal-effect selfish genetic element drives population replacement in *Drosophila*. *Science* 316, 597–600.

Curtis, C.F. (1968) Possible use of translocations to fix desirable genes in insect pest populations. *Nature* 218, 368–369.

Davis, S., Bax, N., and Grewe, P. (2001) Engineered underdominance allows efficient and economical introgression of traits into pest populations. *Journal of Theoretical Biology* 212, 83–98.

Dearden, P.K., Gemmell, N.J., Mercier, O.R., Lester, P.J., Scott, M.J. *et al.* (2017) The potential for the use of gene drives for pest control in New Zealand: a perspective. *Journal of the Royal Society of New Zealand* 48, 1–20.

Dhole, S., Lloyd, A.L. and Gould, F. (2019) Tethered homing gene drives: a new design for spatially restricted population replacement and suppression. *Evolutionary Applications* 12, 1688–1702.

Dhole, S., Lloyd, A.L. and Gould, F. (2020) Gene drive dynamics in natural populations: the importance of density dependence, space, and sex. *Annual Review of Ecology, Evolution, and Systematics* 51, 505–531.

DiCarlo, J.E., Chavez, A., Dietz, S.L., Esvelt, K.M. and Church, G.M. (2015) Safeguarding CRISPR-Cas9 gene drives in yeast. *Nature Biotechnology* 33, 1250–1255.

Eckhoff, P.A., Wenger, E.A., Godfray, H.C. and Burt, A. (2017) Impact of mosquito gene drive on malaria elimination in a computational model with explicit spatial and temporal dynamics. *Proceedings of the National Academy of Sciences USA* 114, e255–e264.

Esvelt, K.M., Smidler, A.L., Catteruccia, F. and Church, G.M. (2014) Concerning RNA-guided gene drives for the alteration of wild populations. *Elife* 3, e03401.

Faber, N.R., McFarlane, G.R., Gaynor, R.C., Pocrnic, I., Whitelaw, C.B.A. and Gorjanc, G. (2021) Novel combination of CRISPR-based gene drives eliminates resistance and localises spread. *Scientific Reports* 11, 3719.

Fasulo, B., Meccariello, A., Morgan, M., Borufka, C., Papathanos, P.A. and Windbichler, N. (2020) A fly model establishes distinct mechanisms for synthetic CRISPR/Cas9 sex distorters. *PLoS Genetics* 16, e1008647.

Foster, G.G., Whitten, M.J., Prout, T. and Gill, R. (1972) Chromosome rearrangements for the control of insect pests. *Science* 176, 875–880.

Galizi, R., Doyle, L.A., Menichelli, M., Bernardini, F., Deredec, A. *et al.* (2014) A synthetic sex ratio distortion system for the control of the human malaria mosquito. *Nature Communications* 5, 3977.

Galizi, R., Hammond, A., Kyrou, K., Taxiarchi, C., Bernardini, F. *et al.* (2016) A CRISPR-Cas9 sex-ratio distortion system for genetic control. *Science Reports* 6, 31139.

Gantz, V.M. and Bier, E. (2015a) The dawn of active genetics. *Bioessays* 38, 50–63.

Gantz, V.M. and Bier, E. (2015b) Genome editing. The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. *Science* 348, 442–444.

Gantz, V.M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V.M. *et al.* (2015) Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. *Proceedings of the National Academy of Sciences USA* 112, E6736–E6743.

Girardin, L., Calvez, V. and Débarre, F. (2019) Catch me if you can: a spatial model for a brake-driven gene drive reversal. *Bulletin of Mathematical Biology* 81, 5054–5088.

Gould, F., Huang, Y., Legros, M. and Lloyd, A.L. (2008) A killer-rescue system for self-limiting gene drive of anti-pathogen constructs. *Proceedings of the Royal Society B: Biological Sciences* 275, 2823–2829.

Grunwald, H.A., Gantz, V.M., Poplawski, G., Xu, X.S., Bier, E. and Cooper, K.L. (2019) Super-Mendelian inheritance mediated by CRISPR–Cas9 in the female mouse germline. *Nature* 566, 105–109.

Guichard, A., Haque, T., Bobik, M., Xu, X.-R.S., Klanseck, C. *et al.* (2019) Efficient allelic-drive in *Drosophila*. *Nature Communications* 10, 1640.

Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C. *et al.* (2015) A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nature Biotechnology* 34, 78–83.

Hammond, A., Karlsson, X., Morianou, I., Kyrou, K., Beaghton, A. *et al.* (2021) Regulating the expression of gene drives is key to increasing their invasive potential and the mitigation of resistance. *PLoS Genetics* 17, e1009321.

Hay, B.A., Oberhofer, G. and Guo, M. (2021) Engineering the composition and fate of wild populations with gene drive. *Annual Review of Entomology* 66, 407–434.

Huang, Y., Lloyd, A.L., Legros, M. and Gould, F. (2011) Gene-drive into insect populations with age and spatial structure: a theoretical assessment. *Evolutionary Applications* 4, 415–428.

Jones, R.T., Ant, T.H., Cameron, M.M. and Logan, J.G. (2021) Novel control strategies for mosquito-borne diseases. *Philosophical Transactions of the Royal Society B* 376, 20190802.

Kandul, N.P., Liu, J., Buchman, A., Gantz, V.M., Bier, E. and Akbari, O.S. (2020) Assessment of a split homing based gene drive for efficient knockout of multiple genes. *G3: Genes, Genomes, Genetics* 10, 827–837.

Kandul, N.P., Liu, J., Bennett, J.B. ,Marshall, J.M. and Akbari, O.S. (2021) *A confinable home-and-rescue gene drive for population modification.* eLife 10, e65939.

Kyrou, K., Hammond, A.M., Galizi, R., Kranjc, N., Burt, A. *et al.* (2018) A CRISPR-Cas9 gene drive targeting *doublesex* causes complete population suppression in caged *Anopheles gambiae* mosquitoes. *Nature Biotechnology* 36, 1062–1066.

Langmüller, A.M., Champer, J., Lapinska, S., Xie, L., Liu, J. *et al.* (2021) Fitness effects of CRISPR endonucleases in *Drosophila melanogaster* populations. *bioRxiv*.

Leftwich, P.T., Edgington, M.P., Harvey-Samuel, T., Carabajal Paladino, L.Z., Norman, V.C. and Alphey, L. (2018) Recent advances in threshold-dependent gene drives for mosquitoes. *Biochemical Society Transactions* 46, 1203–1212.

Li, F. and Scott, M.J. (2016) CRISPR/Cas9-mediated mutagenesis of the white and Sex lethal loci in the invasive pest, *Drosophila suzukii*. *Biochemical and Biophysical Research Communications* 469, 911–916.

Li, M., Yang, T., Kandul, N.P., Bui, M., Gamez, S. *et al.* (2020) Development of a confinable gene drive system in the human disease vector *Aedes aegypti. eLife* 9, e51701.

Liu, J., Champer, J., Langmüller, A.M., Liu, C., Chung, J. *et al.* (2019) Maximum likelihood estimation of fitness components in experimental evolution. *Genetics* 211, 1005–1017.

López Del Amo, V., Bishop, A.L., Sánchez, C H.M., Bennett, J.B., Feng, X. *et al.* (2020a) A transcomplementing gene drive provides a flexible platform for laboratory investigation and potential field deployment. *Nature Communications* 11, 1–12.

López Del Amo, V., Leger, B.S., Cox, K.J., Gill, S., Bishop, A.L. *et al.* (2020b) Small-molecule control of super-Mendelian inheritance in gene drives. *Cell Reports* 31, 107841.

Lorimer, N., Hallinan, E. and Rai, K.S. (1972) Translocation homozygotes in the yellow fever mosquito, *Aedes aegypti*. *The Journal of Heredity* 63, 158–66.

Macias, V.M., Ohm, J.R. and Rasgon, J.L. (2017) Gene drive for mosquito control: where did it come from and where are we headed? *International Journal of Environmental Research and Public Health* 14, 1006.

Maselko, M., Feltman, N., Upadhyay, A., Hayward, A., Das, S. *et al.* (2020) Engineering multiple species-like genetic incompatibilities in insects. *Nature Communications* 11, 1–7.

Mérel, V., Boulesteix, M., Fablet, M. and Vieira, C. (2020) Transposable elements in *Drosophila*. *Mobile DNA* 11, Article 23.

Metzloff, M., Yang, E., Dhole, S., Clark, A.G., Messer, P.W. and Champer, J. (2022) Experimental demonstration of tethered gene drive systems for confined population modification or suppression. *BMC Biology* 20, 2022. *bioRxiv* 2021.05.29.446308.

Noble, C., Min, J., Olejarz, J., Buchthal, J., Chavez, A. *et al.* (2019) Daisy-chain gene drives for the alteration of local populations. *Proceedings of the National Academy of Sciences USA* 116, 8275–8282.

North, A.R., Burt, A. and Godfray, H.C.J. (2019) Modelling the potential of genetic control of malaria mosquitoes at national scale. *BMC Biology* 17, 26.

North, A.R. Burt, A. and Godfray, H.C.J. (2020) Modelling the suppression of a malaria vector using a CRISPR-Cas9 gene drive to reduce female fertility. *BMC Biology* 18, 98.

Oberhofer, G. Ivy, T. and Hay, B.A. (2018) Behavior of homing endonuclease gene drives targeting genes required for viability or female fertility with multiplexed guide RNAs. *Proceedings of the National Academy of Sciences USA* 115, E9343–E9352.

Oberhofer, G. Ivy, T. and Hay, B.A. (2019) Cleave and Rescue, a novel selfish genetic element and general strategy for gene drive. *Proceedings of the National Academy of Sciences USA* 116, 6250–6259.

Oberhofer, G. Ivy, T. and Hay, B.A. (2020) Gene drive and resilience through renewal with next generation Cleave and Rescue selfish genetic elements. *Proceedings of the National Academy of Sciences USA* 117, 9013–9021.

Oberhofer, G. Ivy, T. and Hay, B.A. (2021) Split versions of Cleave and Rescue selfish genetic elements for measured self limiting gene drive. *PLoS Genetics* 17, e1009385.

Port, F. and Bullock, S.L. (2016) Augmenting CRISPR applications in *Drosophila* with tRNA-flanked sgRNAs. *Nature Methods* 13, 852–854.

Prowse, T.A. Adikusuma, F., Cassey, P., Thomas, P. and Ross, J. V. (2019) A Y-chromosome shredding gene drive for controlling pest vertebrate populations. *eLife 8*, e41873.

Quinn, C.M. and Nolan, T. (2020) Nuclease-based gene drives, an innovative tool for insect vector control: advantages and challenges of the technology. *Current Opinion in Insect Science* 39, 77–83.

Reeves, R.G., Bryk, J., Altrock, P.M., Denton, J.A. and Reed, F.A. (2014) First steps towards underdominant genetic transformation of insect populations. *PLoS ONE* 9, e97557.

Ross, P.A., Turelli, M. and Hoffmann, A.A. (2019) Evolutionary ecology of *Wolbachia* releases for disease control. *Annual Review of Genetics* 53, 93–116.

Scott, M.J., Gould, F., Lorenzen, M., Grubbs, N., Edwards, O. and O'Brochta, D. (2018) Agricultural production: assessment of the potential use of Cas9-mediated gene drive systems for agricultural pest control. *Journal of Responsible Innovation* 5, S98–S120.

Shapiro, R.S., Chavez, A., Porter, C.B.M., Hamblin, M., Kaas, C.S. *et al.* (2018) A CRISPR–Cas9-based gene drive platform for genetic interaction analysis in *Candida albicans*. *Nature Microbiology* 3, 73–82.

Shropshire, J.D. and Bordenstein, S.R. (2019) Two-By-One model of cytoplasmic incompatibility: Synthetic recapitulation by transgenic expression of *cifA* and *cifB* in *Drosophila*. *PLoS Genetics* 15, e1008221.

Simoni, A., Siniscalchi, C., Chan, Y.S., Huen, D.S., Russell, S. *et al.* (2014) Development of synthetic selfish elements based on modular nucleases in *Drosophila melanogaster*. *Nucleic Acids Research* 42, 7461–7472.

Teem, J.L., Alphey, L., Descamps, S., Edgington, M.P., Edwards, O. *et al.* (2020) Genetic biocontrol for invasive species. *Frontiers in Bioengineering and Biotechnology* 8, 452.

Terradas, G., Buchman, A.B., Bennett, J.B., Shriner, I., Marshall, J.M. *et al.* (2021) Inherently confinable split-drive systems in *Drosophila*. *Nature Communications* 12, 1–12.

Verkuijl, S.A.N., Gonzalez, E., Li, M., Ang, J., Kandul, N.P. *et al.* (2020) A CRISPR endonuclease gene drive reveals two distinct mechanisms of inheritance bias. *bioRxiv*.

Webster, S.H., Vella, M.R. and Scott, M.J. (2020) Development and testing of a novel killer–rescue self-limiting gene drive system in *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences* 287, 20192994.

Whitten, M.J. (1971) Insect control by genetic manipulation of natural populations. *Science* 171, 682–684.

Windbichler, N., Papathanos, P.A. and Crisanti, A. (2008) Targeting the X chromosome during spermatogenesis induces Y chromosome transmission ratio distortion and early dominant embryo lethality in *Anopheles gambiae*. *PLoS Genetics* 4, e1000291.

Xu, X.S., Bulger, E.A., Gantz, V.M., Klanseck, C., Heimler, S.R. *et al.* (2020) Active genetic neutralizing elements for halting or deleting gene drives. *Molecular Cell* 80, 246–262.

Yan, Y. and Finnigan, G.C. (2019) Analysis of CRISPR gene drive design in budding yeast. *Access Microbiology* 1, e000059.

Yang, E., Metzloff, M., Langmüller, A.M., Clark, A.G., Messer, P.W. and Champer, J. (2022) A homing suppression gene drive with multiplexed gRNAs maintains high drive conversion efficiency and avoids functional resistance alleles. G3 *Genes|Genomes|Genetics* 12(6), jkac081. 10.1093/g3journal/jkac081.

10 Sex Ratio Manipulation Using Gene Drive for Mosquito Population Control

Yael Arien, Yehonatan Alcalay, Daniella An Haber, Lee Benjamin Lamdan, Rotem Daniel Avraham and Philippos Aris Papathanos*
Department of Entomology, Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot 7610001, Israel

10.1 Introduction

Applications of genetic control of harmful insect species, especially disease vectors and agricultural pests that pose considerable economic and epidemiological risks, have attracted significant attention in recent years given their promise as an alternative, environmentally friendly insect control method. The most common approach has been the mass release of males that are sterilized using radiation (sterile insect technique (SIT)), or more recently infected with *Wolbachia* or modified by transgenes (Alphey, 2014; Zhang *et al.*, 2015; Zheng *et al.*, 2019). When wild monandrous (mating once in their lifetime) females mate with sterile males, their eggs are fertilized by sperm with induced modifications (e.g. mutations or transgenes) that abort embryo development. The earliest successful attempt to apply SIT against insects was conducted by Knipling (1955) and colleagues, who mass released sexually sterilized insects to diminish populations of the screwworm *Cochliomyia hominvorax* over large areas of the USA (Bushland *et al.*, 1955; Krafsur *et al.*, 1986; Krafsur *et al.*, 1987) (see Scott *et al.*, Chapter 17, this volume).

The success of these experiments initiated a 'golden age' of insect genetic control (Gould and Schliekelman, 2004). A number of highly successful area-wide programmes were carried out to demonstrate that such strategies are species-specific and environmentally non-polluting and can serve as an alternative to established methods using insecticides or habitat eradication (Knipling 1955; Krafsur *et al.*, 1986; Alphey *et al.*, 2010). However, for a number of insects, including several mosquito species, the area-wide use of SIT-based strategies has been largely unsuccessful, for example at large regional/national levels. The main challenges faced include the difficulty of sustaining the necessary ratio of sterile to wild males that need to be released over long enough periods and the migration of wild individuals from neighbouring non-targeted areas (Dietz, 1976; Prout, 1978; Bellini *et al.*, 2013a; Balatsos *et al.*, 2021). Practical issues relating to the fitness of males sterilized by ionizing radiation or chemicals also hampered further advancements (Andreasen and Curtis, 2005; Bellini *et al.*, 2013b). During the 1960s and 1970s, research focused on the use of natural sterility (hybrid sterility or cytoplasmic incompatibility), translocations,

*Corresponding author, email: p.papathanos@mail.huji.ac.il

meiotic drive or conditional lethal traits (Whitten, 1985). Although significant progress was made, rarely did this effort translate into truly large-scale implementations, due to the difficulty of establishing and maintaining insects with the required characteristics (Schliekelman et al., 2005).

This chapter will focus on recent progress in applications of modern molecular and genetic techniques to manipulate the sex ratio of mosquito species for the purposes of genetic control. To clarify, sex ratio manipulation is said to occur when, within the pool of an individual's fertile offspring, one of the two sexes is over-represented. We first describe the main approaches for building synthetic sex ratio distorters operating through sex chromosome targeting during spermatogenesis. Next, we discuss how new insights in the sex determination mechanisms of insects can help extend our current arsenal of genetic control strategies and tools.

10.2 Overview and General Principles of Sex Ratio Distorting (SRD) Methods

Sex ratio distorting (SRD) alleles have been proposed to suppress populations of sexually reproducing organisms via extinction of one of the two sexes, which in turn diminishes the population's fertility. Since the overall 'fertility' of a population is normally determined by the fertility and number of its females, which are limited in gamete production, alleles are typically designed to bias the sex ratio towards males. Moreover, females transmit fatal diseases (such as dengue and malaria) and contribute more than males to agricultural losses in disease vectors and agricultural pests, respectively. Mathematical modelling and cage suppression experiments have shown that the use of SRD alleles is significantly more efficient than SIT when considering the number of insects that need to be released (Schliekelman et al., 2005; Burt and Deredec 2018; Pollegioni et al., 2020). This is because transgenic male offspring can maintain the SRD alleles in the population for a certain time period, even when releases are halted.

Hamilton (1967) was the first to propose that SRD alleles could be applied to eradicate mosquito populations by imposing 'extraordinary' sex ratios in a population. He considered a population in which males are the heterogametic sex (XY), thus the relative sex ratio is dependent on the ratio of X chromosome sperm to Y chromosome sperm being used for fertilizations. He showed that mutant Y chromosomes that can bias fertilizations in their favour, such that a male only produces sons, gain a selective advantage that allows them to spread within the population. In the absence of resistance against the novel mutation, the mutated Y chromosome could eliminate the X chromosome and eventually lead to population collapse due to the lack of females. Because this bias arises through unequal gametogenesis in the parent prior to fertilization, it does not result in a reduction of fecundity of the male, allowing the mutated Y to invade the population.

In the next section, we describe naturally occurring meiotic drive systems and highlight synthetic versions of these that are now being engineered to target the paternal X chromosome, resulting in sex ratio manipulation. In theory, synthetic distorters have the advantage that they would be unaffected by already widespread resistance alleles, counteracting natural drive systems. In other words, they should be more evolutionarily resilient and simpler to build, apply and monitor. We also examine sex ratio distorting mechanisms that act post-zygotically, when the survival of one of the sexual fates is selectively diminished.

10.3 Meiotic Drive and Engineered X-Chromosome Shredders

Meiotic drive systems alter the normal process of meiosis in a way that one allele out of a gametic pool is over-represented in the subsequent generations (Zimmering et al., 1970). If the alternative allele is equally represented in the fertilizing gametic pool, even if these do not result in viable offspring, then the effect of segregation distortion will not result in meiotic drive. In this case,

higher representation of the altered allele in the next generation is caused by a fecundity loss for the parent, rather than from a net gain in fertilization events (such a scenario is related to post-zygotic X-poisoning strategies discussed in the next section). At the population level, a meiotic drive may increase in frequency in spite of deleterious physiological effects (Hamilton, 1967). When segregation distorters (SD) are physically linked to sex-determining loci or sex chromosomes, meiotic drive will result in an unequal distribution of sexes in the next generation.

The phenomenon of meiotic drive was first described in detail in *Drosophila melanogaster*, when workers measuring the fitness of second chromosomes taken from wild populations identified *SD*. *SD* has become the most intensively studied example of meiotic drive with more than 50 years of work elucidating its underlying biology (Brand *et al.*, 2015). Sex-linked SRD is more common in systems with male heterogamety, and usually it is the X chromosome that drives against the Y. Since recombination between sex chromosomes of heteromorphic males is already greatly reduced or eliminated, sex chromosomes are well-suited genomic sites for meiotic drive systems to inhabit, and indeed sex chromosome SRDs are over-represented in nature (Hammer, 1991; Lyttle, 1991).

Cases of Y-linked SRD in insects have been documented in culicine mosquitoes. Interestingly, both mosquito species that harbour these sex ratio distorters, *Aedes aegypti* and *Culex pipiens*, actually have homomorphic sex chromosomes on chromosome 1 (Lyttle, 1991). Males are heterozygous at the sex-determining locus *Mm* and females represent the homozygous *mm* condition. The meiotic drive locus only functions when it is located in *cis* to *M* and is denoted as the M^D gene. M^D acts in *trans* on a responder locus that is proximal to and indistinguishable from *m*. The sensitivity level of the *m*-bearing chromosome to M^D varies widely from sensitive (m^s) to insensitive (m^i) (Wood and Newton, 1991; Cha *et al.*, 2006). Cytological studies have shown that the male bias is associated with preferential breakage of chromosomes bearing m^s alleles during the early meiotic stages of spermatogenesis, which results in a decrease in female progeny (Newton *et al.*, 1974; Sweeny and Barr, 1978). During the 1970s trials were initiated to assess its suitability for controlling natural populations of this mosquito using the M^D locus. These experiments revealed the swiftness with which resistance to M^D was selected for in females of cage populations, as predicted by Hamilton (1967). Moreover, the level of distortion ultimately attained was insufficient to achieve effective population control (Hickey and Craig, 1966; Robinson, 1983).

Work in *Anopheles gambiae* mosquitoes, which have heteromorphic XY sex chromosomes, is now being pursued with the long-term goal of inserting on the Y chromosome a transgene that can specifically destroy the X chromosome during male meiosis, inspired by the initial cytological observations describing M^D. The system under consideration relies on the expression of a Y-linked endonuclease that can cleave DNA sequences (15–30 bp) that are uniquely present on the X chromosome (Burt, 2003). Expression of such an endonuclease during male meiosis would lead to recognition and subsequent 'shredding' of the X chromosome, such that X-bearing sperm, which ordinarily give rise to daughters, are eliminated during spermatogenesis (Fig. 10.1). Previous work has shown that *An. gambiae* lends itself for the development of such a system on the basis of the genomic organization of its rDNA genes, which are exclusively located on the X chromosome in a tandemly arranged cluster composed of hundreds of copies (Collins *et al.*, 1987, 1989; Paskewitz and Collins, 1990). The opportunity arose in the use of the naturally occurring, well-studied homing endonuclease I-*Ppo*I that evolved to specifically cleave a 29 bp recognition sequence within the peptydil transferase centre of the 28S rDNA gene. To assess whether expression of this endonuclease can selectively cleave *An. gambiae* X chromosome, transgenic lines were generated in which expression of I-*Ppo*I was driven from regulatory regions of the spermatogenesis-specific *β2-tubulin* gene (Windbichler *et al.*, 2008). This promoter
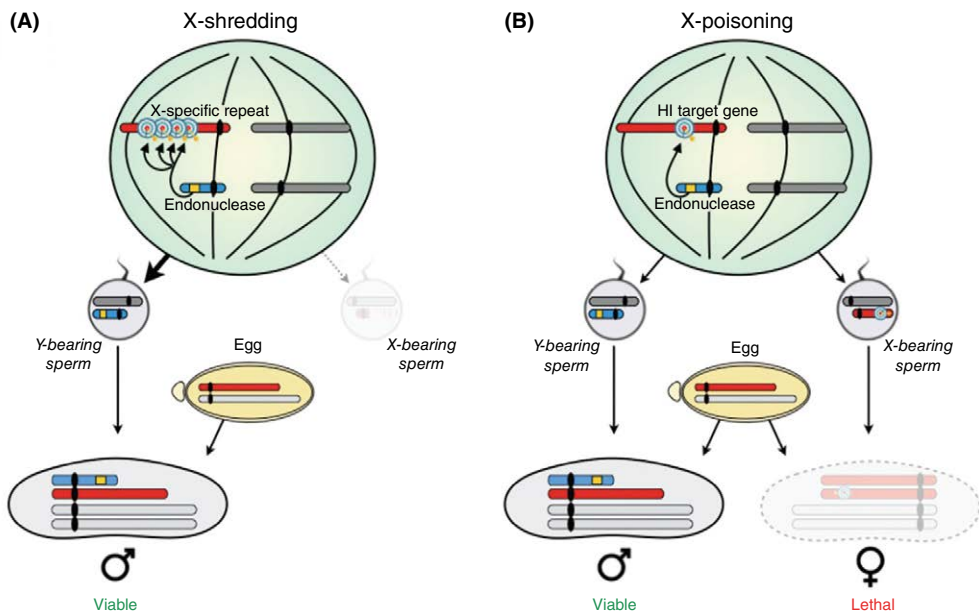
Fig. 10.1. Schematic of Y-linked X-shredding and X-poisoning sex ratio distorters. (A) In X-shredding, CRISPR/Cas9 'shred' a highly repetitive X chromosome-specific sequence in X-bearing sperm, resulting in their absence among successfully fertilizing spermatozoa (*pre-zygotic effect*). **(B)** X-poisoning is based on targeting X-linked haplo-insufficient genes HI (for example, ribosomal protein genes) during spermatogenesis, resulting in dominant lethality of daughters during development (*post-zygotic effect*).

was chosen as it had already been shown to drive expression of transgenes during male meiosis in a number of insect species (Catteruccia *et al.*, 2005; Smith *et al.*, 2007; Scolari *et al.*, 2008; Zimowska *et al.*, 2009). Given the rarity of Y chromosome integrations and the complexity of expression from this chromosome, transgenic constructs were initially assessed in autosomal locations. Autosomal integrations would be expected to display distortions in the inheritance of the sex chromosomes if sperm harbouring shredded X chromosomes were incapacitated. Nonetheless, the construct itself, not being bound to the Y chromosome, would not directly benefit from the deviations and would thus not display meiotic drive (Fig. 10.2A). Surprisingly, transgenic I-*Ppo*I males induced dominant embryonic lethality in their offspring, which rarely progressed beyond the cellularization stage of the embryo (Windbichler *et al.*, 2008). However, when the underlying sex ratio of the inviable eggs was assessed using markers specific to the Y chromosome, it became

clear that, underlying the embryonic lethality, sex ratio distortion was actually occurring (90% males in F_1). The embryonic lethality phenotype was linked to activity of the I-*Ppo*I endonuclease, carried over in sperm against the maternal X chromosomes in the fertilized embryos (Windbichler *et al.*, 2008).

To address the embryonic lethality and to generate true sex ratio distorters, Galizi *et al.* (2014) restricted the activity of the cytotoxic I-*Ppo*I endonuclease during spermatogenesis. Of all the transgenic strains examined, gfp124L-2, since renamed by Target Malaria as Ag(PMB)1 (for *An. gambiae* Paternal Male Bias strain 1) expressing the I-*Ppo*I structural variant W124L, produced about 95% male offspring, without significantly impairing male fertility (Galizi *et al.*, 2014). They also demonstrated that these transgenic males can efficiently suppress caged wild-type populations. Overall, the findings of this study set the stage for the foundation of a novel genetic control strategy based on X-shredding and the possibility

(A)   X-shredding   **(B)**   X-poisoning
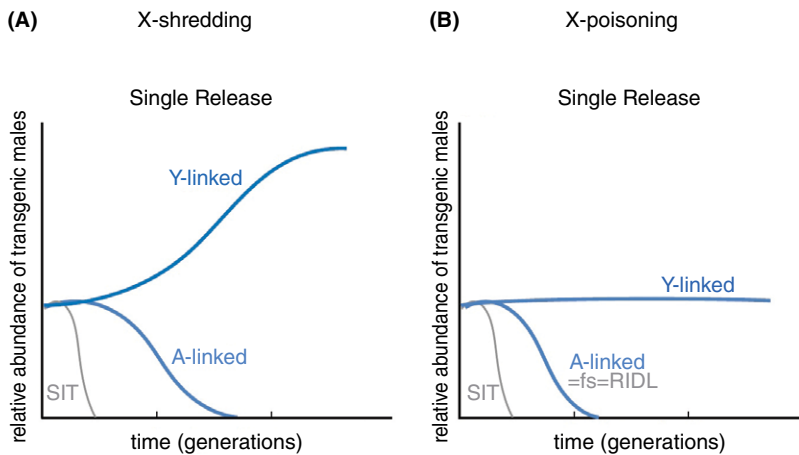
Fig. 10.2. Schematic time course model predicting the population dynamics of X-shredding.
(A) X-poisoning and **(B)** SIT systems expressed from the autosome (A-linked) and from the Y chromosome (Y-linked) following a single release. (Adapted from Burt and Deredec, 2018.)

to build Y-chromosome drives (discussed below). Importantly, when located on autosomes, X-shredders like Ag(PMB)1 are not designed for gene drive in their current form; they neither benefit from the distortion of sex chromosomes during meiosis, nor do they display any fitness advantage over wild-type mosquitoes. Therefore, despite being more efficient than SIT and fs-RIDL (female-specific release of insect carrying dominant lethals) (see Scott *et al.*, Chapter 17; Handler and Schetelig, Chapter 21; Morrison, Chapter 23, this volume), because they operate pre-zygotically to eliminate females at the sperm level, models predict that autosomal X-shredders would ultimately disappear over time, when releases are discontinued (Burt and Deredec, 2018) (Fig. 10.2B). Indeed, recent large-cage experiments confirmed the loss of the transgene from the population over time (Pollegioni *et al.*, 2020). Unfortunately, in the vast majority of insects, rDNA genes are not situated exclusively on the X chromosome, meaning that the use of the I-*Ppo*I endonuclease is not likely to be portable across species.

    The development of the CRISPR system opened a broad set of opportunities for genome editing, given its high flexibility. Since X-shredding exploits the near-universal significance of paternal chromosome inheritance on the outcome of the sex of an individual,

developing CRISPR-based X-shredders in other insect pests was possible. There were five essential requirements required to do so: (i) an XY male karyotype; (ii) genetic transformation; (iii) regulatory elements (promoters) that can drive expression of the X-shredding nuclease during spermatogenesis; (iv) an endonuclease platform, such as the CRISPR/Cas9 system, that can be directed against X-chromosome-specific sequences; and finally (v) the existence of sequences on the X chromosome that are both specific and abundant to it. To test whether CRISPR/Cas9-based X-shredders could be engineered, single guide RNAs (sgRNAs) were first designed to target the same *An. gambiae* ribosomal rDNA genes targeted by the I-*Ppo*I X-shredder. A target sequence downstream of the original site was selected, since this sequence was only conserved within the *An. gambiae* complex (Galizi *et al.*, 2016), versus the I-*Ppo*I target site that exists in all eukaryotes. Expression of Cas9 during spermatogenesis from autosomal constructs containing the same *beta2-tubulin* promoter driving the endonuclease led to shredding of the X chromosome, resulting in male bias among progeny (86.1–94.8%). This occurred without impairing male fertility resulting from paternal sperm carryover, similar to the wild-type I-*Ppo*I, and the levels of sex distortion were similar to those used

for the engineered I-*Ppo*I variants (Galizi *et al.*, 2014). These results confirmed that the CRISPR system could be successfully adapted for X-shredder development in other mosquito species, or even within *An. gambiae* or closely related species through the targeting of X-chromosome sequences that are genus- or complex- or species-specific. Doing so would also provide opportunities to explore more broadly the characteristics of what constitutes an ideal CRISPR-based X-shredding target sequence in terms of copy number, distribution and position on the X-chromosome, relevance of function, etc.

However, knowledge of naturally occurring, multi-copy, X-specific sequences, such as X-specific satellite DNA, is complicated, because such repetitive DNA sequences are *ipso facto* excluded from genome assemblies. Furthermore, few studies deal with such elements, particularly in non-model organisms, because of the difficulty of studying and manipulating repetitive DNA sequences. Indeed, even after 21 years since the publication of the first genome assembly of *An. gambiae* (Holt *et al.*, 2002), the rDNA cluster is still not correctly represented and assembled in the current genome assembly. The original wisdom of the rDNAs' specificity to the X chromosome in *An. gambiae* came from early studies of mosquito population genetics using cytology. To overcome this limitation, we previously developed a computational pipeline, redkmer, specifically for CRISPR-based X-shredders (Papathanos and Windbichler, 2018). Redkmer uses raw whole genome sequencing (WGS) data and identifies among the data sequences that are abundant, likely to be X chromosome-specific, and targetable by CRISPR endonucleases like Cas9. In tests of redkmer using *An. gambiae* data, the pipeline correctly identified target sites from the rDNA locus and physically linked rDNA-specific repetitive elements as suitable for X-shredding. Leveraging available WGS data and redkmer, we have since developed X-shredders in two non-mosquito species, namely *D. melanogaster* (Fasulo *et al.*, 2020; Meccariello *et al.*, 2021) and the agricultural fruit fly pest *Ceratitis capitata* (Meccariello *et al.*, 2021). Unlike the high levels of male bias that were achieved in *An. gambiae* using both the I-*Ppo*I and the Cas9 autosomal X-shredders, however, in both species sex bias was much less pronounced (61.5% and about 80% for *D. melanogaster* and *C. capitata*, respectively). In both cases, the target sites were selected for being the most abundant and specific to the X chromosome in both species, respectively. In *D. melanogaster* the top-ranking sites were located in annotated genes of the X chromosome, whereas in *C. capitata* the top-ranking sites were embedded in repeats composed of fragmented retrotransposons and simple repeat elements, while distortion was not as high as was originally developed in *An. gambiae*. These studies clearly demonstrated that X-shredders can be translated to work in other species by targeting both genes and resident repeats of the X chromosome, and these repeats do not have to be highly abundant, conserved or functional. Notably, in all engineered SRDs discussed here, expression of X-shredders was done from autosomal insertions with the *beta2-tubulin* promoter driving expression of the endonuclease.

We continue the X-shredder story in section 10.5 below, where we discuss the steps required and progress made to date in linking X-shredders to mosquito Y chromosomes. This represents the next and final step required in building true meiotic drives that benefit from the distortion generated, and ensuring that all male descendants contain the X-shredder to bias future generation (Fig. 10.2A). Next, we will discuss engineering non-invasive SRDs, in which many of the same rules to engineering apply, with small changes that can have a dramatic impact on the outcomes of sex distortion on the population and the types of genetic control strategies that can be developed.

10.4 Post-Zygotic Sex Distortion Through Sex-Specific Lethality

As discussed above, Y-linked SRDs can form the basis of a self-sustaining genetic control strategy. By being linked to the Y chromosome, an X-shredder SRD is both insulated

from selection and benefits from its increased transmission (preferential inheritance compared with wild-type Y chromosomes). This advantage ultimately allows it to rapidly invade the population, skewing sex ratios towards males until population extinction occurs or resistance alleles arise. However, under some scenarios the use of an invasive SRD may not be desirable. For example, one might want to suppress a localized outbreak of an insect pest species in a newly invaded area without harming insects in its native range.

In this case SRDs can be developed to skew sex ratios post-zygotically, through the targeted killing of field-born daughters. In most designs this results in a self-limiting (i.e. non-invasive) construct that can be significantly more practical or efficient than the classical SIT (Schliekelman *et al.*, 2005). One successful approach has been the use of transgenic constructs that conditionally and specifically result in female lethality, without affecting male fitness (see Scott *et al.*, Chapter 17; Handler and Schetelig, Chapter 21; Morrison, Chapter 23, this volume). Transgenic field-born males effectively amplify the effect of insect release by inducing lethality in their daughters that inherit the transgene. The most successful demonstration of this system, fs-RIDL, was developed by the UK-based biotech firm Oxitec. Field trials have already been conducted for *Ae. aegypti* in the Cayman Islands, Malaysia, Brazil and recently also in the Florida Keys, USA (Harris *et al.*, 2012, 2011; Lacroix *et al.*, 2012; Carvalho *et al.*, 2015b; Waltz, 2021) and had also been developed in *Aedes albopictus* (Labbé *et al.*, 2012). This strategy is more effective than SIT, while still having geographically restricted impacts (Fig. 10.2).

More recently, Burt and Deredec (2018) used modelling to show that a Y-linked construct, acting during sperm development to induce mutations that result in dominant post-zygotic killing of daughters, theoretically represents the most efficient self-limiting strategy that can be engineered. A number of potential molecular designs were proposed, but for the purpose of this chapter we consider one design, called X-poisoning, for simplicity. Unlike X-shred-

ders, here X-chromosome targeting does not affect the relative fertilization success of X-bearing gametes, but is instead restricted to the mutagenesis of an X-linked target gene that has a haplo-insufficient (HI) or haplo-sterile phenotype. Mutated alleles are transmitted exclusively to daughters by virtue of their X-linkage and, assuming mutagenesis results in a null allele of the HI gene, female offspring will be inviable. Male descendants inherit the paternal Y-linked transgene and a single wild-type maternal allele, which is hyper-transcribed through dosage compensation. Alternatively, autosomal HI genes could also be targeted, but these would need to be essential only for female fitness. With either design, linkage of the construct to the Y chromosome provides the transgene with insulation from the harm it causes to female descendants, which do not inherit the 'Y-linked cause' for their fitness reduction. Since activity of the construct does not result in a net increase in the frequency of Y-bearing gametes (compared with wild-type Y chromosomes) this system is not invasive (self-sustaining) like a Y-linked X-shredder. However, because selection against the Y-linked construct is absent or weak, the dynamics of transgene frequency and population suppression result in a more efficient control strategy than others, for example autosomal X-shredders, RIDL or fs-RIDL. In the idealized case that the Y-linked construct does not impose any additional costs to male fitness, beyond lethality of female descendants, Y-linked X-poisoning alleles can remain in the population even after releases are halted (Fig. 10.2B, shown as a single release). Through this, consecutive releases can act additively to intensify population suppression rather than to maintain it. This represents a marked departure from the classical dynamics of self-limiting constructs and SITs that decrease in frequency and are eventually lost, or more recently gene drives that increase in frequency because of preferential inheritance. Fasulo *et al.* (2020) recently demonstrated proof-of-concept of this in *D. melanogaster*, which they named X-poisoning. Transgenic males expressing autosomal Cas9 from the *beta2-tubulin* promoter and autosomal sgRNAs

targeting X chromosome HI ribosomal protein genes resulted in strong sex ratio distortion (92% males), through female-specific lethality during embryo development. This study confirmed that X-poisoning can be developed by targeting X chromosome-linked genes that have a haplo-insufficient or dominant lethal phenotype using CRISPR, paving the way for developing similar systems in mosquitoes. For X-poisoning, X-linked essential genes will need to be identified and then targeted in new species, which may be more complicated in *Aedes* mosquitoes that have homomorphic sex chromosomes. In this case, targeting genes that have a dominant female-lethal phenotype may be easier (O'Leary and Adelman 2020).

10.5 Engineering Y-linked SRDs in Mosquitoes

In the previous sections we discussed progress in developing synthetic sex ratio distorters for mosquito control. We described the proof-of-concept experiments using autosomal transgenes that demonstrated the potential for systems like X-shredding and X-poisoning. For both of these systems, the next step in the development of these technologies is their transfer to insect Y chromosomes. As we shall see in this section, some progress has been made towards this, but both technical and biological roadblocks remain when transferring constructs to the Y chromosome. We focus initially on technical issues and then discuss species-specific biological roadblocks to technology development. We focus the discussion on species with an XY sex chromosome complement, since most mosquitoes have this arrangement, but many of the principles and technical issues translate to WZ systems also.

Evolutionary theory predicts that the absence of recombination between the X and Y chromosomes ultimately results in a progressive genetic decay of the Y chromosome. Indeed, we now know from studies in *Drosophila* and mammals that Y chromosome evolution is typically characterized by

an erosion of the ancestral gene set and a gradual accumulation of repetitive DNA sequences (Kaiser and Bachtrog, 2010; Bachtrog, 2013; Hughes and Page, 2015). As a consequence of being gene-poor, repeat-rich and (typically) small, mosquito Y chromosomes have mostly been ignored after an early interest in their use as population genetic markers using cytology or PCR. Their repetitive, heterochromatic nature has made them recalcitrant to sequencing methods and genomic analyses, which are designed to handle protein-coding, euchromatic, non-repetitive (or less repetitive) DNA sequences. Therefore, mosquito Y chromosome sequences are relegated to a bin of small, unlocalized scaffolds, which are independent of the assembly coordinate system. Repeats interfere with scaffolding in genome sequencing and with the localization of transgene integrations to chromosomal locations, an outcome from the lack of sufficient distinguishing features in sequenced transgene–chromosomal arm junctions. Y chromosomes do not form polytene structures, also limiting physical mapping. Y chromosome sequences are rapidly evolving at the sequence expression level, and copy-number and Y-encoded genes are often characterized by gigantic introns containing repeats, stretching sequencing chemistries and comparative genomics to their limits (Pertile *et al.*, 2009; Carvalho *et al.*, 2015a). The heterochromatic environment also likely interferes with expression of transgenic constructs, since random integration of transgenes on the Y chromosome are underrepresented even when accounting for chromosome size. Finally, the community of researchers working on mosquito Y chromosome biology and its engineering is very small. Unsurprisingly, genetic and genomic tools for the Y chromosome are slower to be developed compared with resources for autosomal genes or sequences (Hughes and Page, 2015).

Despite these technical challenges, some important progress has been made in recent years towards the engineering of Y chromosomes. Y chromosome transgenic integrations have now been reported in a number of insect species, including *D. melanogaster*,

Ceratitis capitata, *Anopheles* and *Aedes* mosquitoes. For *Drosophila* and *An. gambiae*, these integrations included attP site-specific landing sites enabling secondary integrations into the Y chromosome. The *Anopheles* Y chromosome attP line was generated fortuitously by random integration of a transgene that was subsequently modified using endonuclease-based knock-in to adapt it as a suitable attP landing site (Bernardini *et al.*, 2014). The *Drosophila* Y chromosome attP landing site was generated more recently using a CRISPR/Cas9 knock-in into an annotated Y chromosome sequence of the fly Y chromosome, establishing a pipeline for doing the same in mosquito species (Buchman *et al.*, 2021). For *Ae. aegypti*, multiple transgenics have been isolated over the years that were randomly inserted in proximity to or within the M-locus on chromosome 1, the Y chromosome equivalent (Hall *et al.*, 2015). To the best of our knowledge, there is currently no available attP landing site for secondary integrations in the M-locus, or validated sgRNA target sites that can be used for CRISPR-based knock-in in this species. Importantly, since the M-locus is autosomal, recombination in males can result in breakdown of M-locus linkage. Therefore, not all transgene insertions into the M-locus will be equally good for downstream work – ideally attP landing or sgRNA target sites need to be inserted as close to the male-determining *nix* gene as possible (Buchman *et al.*, 2021).

Important progress has also been made in Y chromosome genomics – a crucial component for the rapid development of synthetic SRDs. The recent advent of third-generation sequencing chemistries that are designed to capture very long nucleotide sequences, like Pacific Biosciences or Oxford Nanopore Technologies, and Hi-C that measures contact between genomic sequences, have significantly improved Y chromosome scaffolding (Hall *et al.*, 2016; Mahajan *et al.*, 2018; Bayega *et al.,* 2020; Bracewell *et al.*, 2020; Ward *et al.*, 2021). Computational methods have also been developed to identify Y-derived sequences, by taking advantage of male specificity and/or differential coverage in whole genome sequencing data between males and females

(Hall *et al.*, 2013; Vicoso and Bachtrog, 2015). As a result, the content of *Anopheles* Y chromosomes and *Aedes* M-loci is now emerging (Hall *et al.*, 2016, 2015), providing also important insights into mosquito sex determination (see below).

One area that remains challenging for the development of Y chromosome-linked SRDs is how to maintain expression and activity once the construct is located on the Y chromosome. Previous studies in *Anopheles* and other XY species have revealed that expression from sex chromosomes during meiotic stages of spermatogenesis is suppressed by a phenomenon known as meiotic sex chromosome inactivation (MSCI) (Magnusson *et al.*, 2012; Vibranovski *et al.*, 2012; Papa *et al.*, 2017; Larson *et al.*, 2018), a timing that unfortunately coincides with the optimal expression of SRDs in male sperm. It has been proposed that MSCI evolved as a side effect of chromatin remodelling that inhibits non-homologous sex chromosomes from recombining (McKee and Handel, 1993). Interestingly, while MSCI is generally conserved in mammals, there may be important differences between insect species: MSCI is likely not possible in *Aedes* and *Culex* mosquitoes because they have homologous sex chromosomes that do recombine. It is likely also to be unnecessary in *Drosophila*, because no recombination happens at male meiosis anyway. Whether MSCI does indeed exist in the *Drosophila* male germline remains debated (Vibranovski, 2014). Evidence for the existence of MSCI in *An. gambiae* was first based on observations that sperm-specific genes are generally underrepresented on the X chromosome (Magnusson *et al.*, 2012; Papa *et al.*, 2017; Larson *et al.*, 2018) and from experimental data exploring the impact of chromosomal position on sperm-specific transgene expression (Galizi *et al.*, 2014; Alcalay *et al.*, 2021). When X-shredders are integrated on autosomes, strong transgene expression and sex ratio distortion is observed. However, when the same constructs are inserted on either the X or Y chromosome, expression of the meiotic X-shredder from the *beta2-tubulin* promoter is not detectable, and the sex ratio of male offspring is 50:50 (Galizi *et al.*, 2014;

Alcalay *et al.*, 2021). Importantly, expression at the same chromosomal position on the Y chromosome (the attP landing site from Bernardini *et al.*, 2014) is not suppressed in somatic tissues or pre-meiotic sperm, since expression of transgenes from the *vasa* and 3xP3 promoters were indistinguishable from autosomal integrations. These data suggest that the occurrence of MSCI in *An. gambiae* is the most significant obstacle to the development of active Y-linked sex ratio distorters. One possible solution might be to express SRDs earlier in spermatogenesis, before MSCI. Care should be taken, however, because earlier expression could result in male sterility – if, for example, X chromosome target genes for X-poisoning are necessary for spermatogenesis. Another intriguing possibility is to learn from evolution itself: how do genes on insect Y chromosomes overcome MSCI? For example, how does the *An. gambiae* Y chromosome YG5 gene overcome MSCI, which was discovered on the Y chromosome as part of an earlier study of its content? Similarly in *Drosophila*, after chromosome pairing, the Y chromosome decondenses and is transcribed throughout the spermatocyte growth period, forming large 'lampbrush loops' (Bonaccorsi *et al.*, 1990; Taxiarchi *et al.*, 2019) in regions of the Y chromosome that contain important male fertility genes.

10.6 Manipulation of Sex Determination Mechanisms

Sex ratios can also be distorted by interfering with the regulatory pathways that orchestrate sex determination. This could be done either to induce, for example, female-specific lethality via targeting of female-specific components of sex determination, or sex conversion to phenotypically alter the sexual fate of an individual. The advantage of sex conversion over female killing is that, like pre-zygotic manipulation, sex ratio manipulation does not come at a cost of reduced fecundity by eliminating half of the progeny. Therefore, instead of killing females, they can be converted to phenotypic males that

can contribute to the spread of the allele; if converted, phenotypic males (PMs) are fertile. Female-to-male conversion has been proposed both as a method to suppress wild populations in the field and as a system to eliminate females from the release generation by converting them into males (effectively doubling instead of halving the release population). Indeed, modelling has shown that, assuming PMs are as fertile as normal genotypic males, such alleles should be more effective in population suppression than both female-killing and SIT (Schliekelman *et al.*, 2005).

Manipulation of sex determination in each species relies on a concrete understanding of the genes involved in the targeted organism. The hierarchical organization of the sex determination pathway in insects is believed to adhere to a similar theme: at the top of the pathway a primary signal leads to the activation of the key gene, which then recruits a conserved double-switch gene that acts diversely in males and females to orchestrate sexual differentiation.

In *D. melanogaster*, where sex determination has been most extensively studied, a double dose of the primary signal, X chromosome-linked signal elements (XSE), initiates female development, while a single dose of XSE leads to male production (Erickson and Quintero, 2007). Thus, XX, XXY and XXYY flies are females, while XY and XO flies are males, and flies with more than two copies of an X chromosome are female but with low viability. In females both X chromosomes remain active, while males compensate for having half the number of X chromosomes as females by roughly doubling the expression levels of X-linked genes, in a process known as dosage compensation (Cline and Meyer, 1996). This primary signal regulates the expression of the master switch gene *Sex lethal* (*Sxl*) early in post-zygotic development. A double dose of the X-linked nominators in females initiates expression of *Sxl* from its early promoter, leading to a burst of SXL protein in female embryos. Later in development, *Sxl* is expressed in both sexes. In males, all *Sxl* transcripts include a translation-terminating third exon. In females, sex-specific splicing is dependent on the early

accumulation of SXL, which then acts to splice primary mRNAs of its own gene. This results in a positive feedback loop that establishes and maintains sexual memory (Cline, 1984; Bell *et al.*, 1991). The activation of *Sxl* in females results in the appropriate splicing of the key gene *transformer* (*tra*), which in turn regulates the alternative splicing of the *doublesex* (*dsx*) and *fruitless* (*fru*) genes, to produce sex-specific transcription factors that ultimately control most aspects of sexual differentiation and behaviour (Shearman, 2002). Since the pathway is turned off in males, default male-specific isoforms of *dsx* and *fru* are produced instead.

Substantial efforts have gone into elucidating similar details of the sex determination pathway in other insects, mostly on the basis of homology to the *Drosophila* model. What has emerged has been the understanding that the evolution of the sex-determining cascade occurs from the 'bottom up' (Wilkins, 1995). Genes at the bottom of the cascade represent older, more ancestral members of the pathway that are more highly conserved between related species. Upstream genes are recruited by frequency-dependent selection for the minority sex at each step, to reverse the sexual choice of the gene they precede (for a theoretical analysis of this model, see Pomiankowski, 2004).

Predictably, therefore, *dsx* has been identified in all Diptera, Lepidoptera and Hymenoptera examined (Shukla and Nagaraju, 2010). In most of these insects, *dsx* is sex-specifically spliced into one male-specific and one female-specific isoform, like in *Drosophila* and *An. gambiae*, whereas in the housefly *Musca domestica*, the honeybee *Apis mellifera*, the silkmoth *Bombyx mori* and the mosquito *Ae. aegypti*, *dsx* is spliced to produce more than two isoforms (Ohbayashi *et al.*, 2001; Hediger *et al.*, 2004; Cho *et al.*, 2007; Salvemini *et al.*, 2011; Shukla *et al.*, 2011). While *dsx* is well conserved at the bottom of the pathway, genes upstream are more divergent.

Transformer (*tra*) orthologues have been identified in the jewel wasp *Nasonia vitripennis*, *M. domestica*, *C. capitata*, the Australian sheep blowfly *Lucilia cuprina*, the olive fruit fly *Bactrocera oleae*, the West Indian fruit fly *Anastrepha obliqua*, the Caribbean fruit fly *Anastrepha suspensa*, the tsetse fly *Glossina morsitans* and *A. mellifera* and in each case translation-terminating male exons are the basis of an autoregulatory splicing mechanism (Inoue and Hiroyoshi, 1986; Pane *et al.*, 2002; Lagos *et al.*, 2007; Beukeboom and van de Zande, 2010; Sarno *et al.*, 2010). In mosquitoes *tra* has not been identified, but the discovery of sex-specific splicing of *dsx* and *fru* in both *Aedes* and *Anopheles* mosquitoes suggests the presence of a *tra*-like activity (Sarno *et al.*, 2010).

The primary signals that initiate sex determination display the most significant natural diversity. In *M. domestica* the male-determining gene has been found linked to either of the autosomes (I–V) or the X or Y chromosomes in isolated populations from different parts of the world (Sakai and Miller, 1992; Kozielska *et al.*, 2008). In Hymenoptera, sex is determined by a haplodiploid mechanism in which males emerge from unfertilized eggs and females from fertilized eggs (Beukeboom and van de Zande, 2010). In silkworm a W-chromosome-linked Piwi-interacting RNA (piRNA) has been linked as the female-determining factor (Kiuchi *et al.*, 2014). In mosquitoes, sex is determined by a dominant male-determining factor (M-factor), which serves as a primary signal. Sufficient expression of these primary genes during the early embryonic phase activated male-specific splicing of *dsx* and *fru* and triggered male development (Hall *et al.*, 2015). Similarly to humans, in *C. capitata* and *An. gambiae*, the presence or absence of the Y chromosome, containing the M-factor, determines sex (Baker and Sakai, 1979; Willhoeft and Franz, 1996; Krzywinski *et al.*, 2004; Beukeboom and van de Zande, 2010). As in other taxa with heterogametic sex chromosomes, the pathway of dosage compensation is often linked to sex determination, meaning manipulation of sex determination genes can impact dosage compensation (see more below). *Aedes* and *Culex* mosquitoes have homomorphic sex-determining chromosomes with a non-recombining sex-determining region containing the M-factor. This region is

commonly called the M-locus and is located on chromosome 1 (Craig and Hickey, 1967; Baker and Sakai, 1979).

The first M-factor in mosquitoes was reported by Hall *et al.* (2015). They discovered and named *nix*, located within the M-locus of *Ae. aegypt*i. They showed that it acts upstream of *dsx* and *fru*, and confers male-specific splicing of those genes. Knockout of *nix* using CRISPR/Cas9 in male embryos resulted in feminization of adult mosquitoes. Moreover, ectopic expression in female embryos causes the development of partially masculinized adults. In a recent study, Aryan *et al.* (2020) showed conversion of genetic females into phenotypic males by overexpressing *nix* from an autosomal transgene. However, due to the absence of another M-locus gene '*myo-sex*' that encodes a male-specific myosin gene needed for male flight, converted individuals were flightless males. Therefore, *nix* is not sufficient for complete sex conversion in *Ae. aegypti*. A recent study in *Ae. albopictus* revealed that *nix* is sufficient for full masculinization, as the converted males were able to fly. Additionally, using a specific fluorescence marker on the transgene construct enabled successful automated sex sorting in this species (Lutrat *et al.*, 2022). Developing homing base gene drive strategies for *nix*, including other needed M-locus genes, could have great potential for population suppression by sex conversion.

M-factors have now also been discovered in *Anopheles* mosquitoes: *Yob* and *Guy1* in *An. gambiae* and *An. stephensi,* respectively. In both species these genes act as a primary signal to sex determination and are linked and expressed from the Y chromosome at an early embryonic stage. Ectopic expression of these genes in early embryos resulted in female-specific lethality (Criscione *et al.*, 2013, 2016; Krzywinska *et al.*, 2016) and embryonic knockdown of *Yob* resulted in the regulation of *dsx* splicing shifting towards female-specific isoforms and caused 100% male mortality during development (Krzywinska *et al.*, 2016). A follow-up study, which included autosomal transgenic strains expressing *Yob* cDNA ectopically, resulted in a strong male bias (Krzywinska and Krzywinski, 2018). However, unlike *nix* in *Ae. aegypti* and *Ae. albopictus*, ectopic expression of *yob* and *guy1* did not cause female-to-male conversion but, instead, led to female-specific lethality. Lethality was most likely due to the involvement of *Yob* and *Guy1* in dosage compensation regulation, causing hyper-expression of the two X chromosomes in genetic females (Rose *et al.*, 2016; Qi *et al.*, 2019). This suggests also that male-specific lethality, in *Yob* knockdown males, is likely a result of insufficient expression of the X chromosome, since *Yob* regulates dosage compensation. *Ae. aegypti,* because it contains homomorphic sex-chromosomes (Hall *et al.*, 2015), likely does not require dosage compensation, making the possibility of sex conversion perhaps easier from an engineering perspective. Therefore, sex conversion in *Anopheles* might only be possible if immediately downstream genes (i.e., functional *tra* orthologues) are not involved in the dosage compensation pathway. On the other hand, manipulation of genes with female-killing properties could be used to conditionally eliminate females for population suppression or for genetic sexing.

Manipulation of the sex determination pathway can also be achieved by interfering directly with genes downstream of the primary signal. Transient injection of double-stranded RNA targeting the *tra* gene has been shown to lead to sex conversion in *D. melanogaster*, as well as *C. capitata*, *B. oleae*, *A. suspensa* and *L. cuprina* (Pane *et al.*, 2002; Lagos *et al.*, 2007; Concha and Scott, 2009; Schetelig *et al.*, 2012). This results in 100% of the progeny being male – 50% genotypic XY males and 50% phenotypic XX males. The fertility status of the phenotypic XX males varies and is species-specific. In *Drosophila*, XX males are infertile because they lack genes on the Y chromosome that are required for male fertility (Hackstein and Hochstenbach, 1995). On the other hand, *Ceratitis* and *Bactrocera* phenotypic males that were generated using transient RNAi were fertile.

A homing-based gene drive system in *D. melanogaster* showed that CRISPR/Cas9-mediated knockout of the *tra* gene could be

effective in female-to-male sex conversion (Carrami *et al*., 2018). Transgenic flies were generated to express a CRISPR/Cas9 element targeting the first exon of *tra* both in somatic tissues, to induce conversion into the male phenotype, and in the germline, to achieve homing and super-Mendelian inheritance. Mathematical models suggest that this system could function as a gene drive in the Mediterranean fruit fly, *C. capitata*, and could be used to suppress its populations by making them increasingly male dominant. However, they also showed a formation of CRISPR/Cas9-resistant alleles at *tra* target sites could ultimately block the drive from spreading. Choosing a conserved target site in homing-based gene drive systems can reduce the occurrence of functional resistance alleles and improves the chances of the driving allele to fixate in the population (Hammond *et al*., 2017). Based on this, a CRISPR-homing gene drive system targeting a highly conserved target site in the *dsx* gene led to the first successful suppression of caged populations using a gene drive in *An. gambiae* mosquitoes (Hammond *et al*., 2017; Kyrou *et al*., 2018). Homozygous mutant females with no functional *dsx* copy were transformed into sterile intersex individuals, while heterozygous females and all males remained fertile and could spread the allele in the population via mating. When combined with autosomal X-shredding, by expressing an additional sgRNA from within the *dsx* gene drive, population suppression can be further enhanced, even though autosomal X-shredding itself is not self-sustaining (Simoni *et al*., 2020).

Other genes involved in the sex determination pathway of *Anopheles* mosquitoes have also been identified. For example, *femaleless* (*fle*) has molecular similarities to the RNA-binding region of TRA2. RNAi knockdown *fle* expression showed decreased female *dsx* transcript and upregulation of X chromosome genes, suggesting it is also involved in the dosage compensation pathway (Simoni *et al*., 2020; Krzywinska *et al*., 2021). Transgenic lines had variable levels of female masculinization and/or female-specific lethality. Interestingly, these lines also suggested that *fle* is a haplo-insufficient gene

and therefore is not suitable as a target in homing-based gene drive systems. The female-specific lethality effect of *fle* could still be used to conditionally eliminate females in *Anopheles* mosquitoes, in a way that resembles female-specific RIDL (Scott, 2021) or postzygotic lethality of females from Y-linked SRDs (Burt and Deredec 2018).

10.7   Conclusions

We have discussed how recent developments in molecular and synthetic biology have provided novel genomic editing tools for sex ratio manipulation and their application to insect population control. We have discussed two SRD approaches using site-specific endonucleases targeting X-bearing gametes during spermatogenesis and how their linkage on the Y chromosome can allow them to suppress populations in a self-sustaining or self-limiting manner.

These proof-of-principle experiments have opened the door for attempts to generate stable transgenes for sex ratio manipulation using gene drive. For the X-shredding and X-poisoning approaches, a method is required to identify sequences repeated exclusively on the X chromosome for the former, and X-linked haplo-insufficient genes for the latter. Linking the SRD trait to the male Y chromosome will help to overcome the obstacle of continuously rearing and releasing transgenic individuals. Given the flexibility of the CRISPR/Cas9 system, it may be possible to test whether those two paradigms, separately or combined, have the potential to become a universal strategy to genetically control a wide variety of insect pests.

Manipulation of genes involved in sex determination causing female-specific lethality and sex conversion provide new ways for population suppression due to the added effect of male bias. The recent progress in molecular mechanisms of sex determination in mosquitoes, combined with CRISPR-based systems, provides new targets and strategies for genetic control for driving male bias into wild populations. Furthermore, producing transgenic lines with all-male progeny (by killing females or complete

sex conversion) could benefit sex separation methods and mass-rearing productivity.

Further studies are needed to shed light on insect sex determination pathways and transcriptome and genome sequences, to improve availability of methodologies to interfere with the expression of genes that specify sex. Future challenges will be to gain more knowledge about the identity and function of sex-determining genes in order to provide novel synthetic SRDs combining homing/nuclease-based gene drives for vector control.

In the mid-1980s Chris Curtis, the most well-known and prolific advocate of applying SIT-based genetic control of mosquitoes, argued that 'there may be a danger that the intellectual appeal of recombinant DNA, transposable elements etc. may lead applied entomologists to waste time on baroque schemes, without thinking whether their aims could be achieved more simply and quickly by old-fashioned selection, translocations etc.' (Curtis, 1985). However, as mentioned before, using the RIDL system with transgenic *Aedes* mosquitoes has already been evaluated in field studies in 2010 in the Cayman Islands, achieving an 80% reduction in the overall population of the target mosquito (Harris *et al.*, 2011, 2012). We have recently witnessed that RIDL male release trials in Brazil led to strong suppression of the wild population (Carvalho *et al.*, 2015b). In a number of agricultural pests, recent advances in sex reversal have been shown in proof-of-principle experiments. The first genetically sterile strain of *A. gambiae* is currently being assessed for its suitability in mass releases of SIT programmes (Windbichler *et al.*, 2008; Klein *et al.*, 2012). Ultimately the future of insect population control may lie in the combination of contemporary molecular biology, transgenic techniques and classical genetics.

Acknowledgement

References

Alcalay, Y., Fuchs, S., Galizi, R., Bernardini, F., Haghighat-Khah, R.E. *et al.* (2021) The potential for a released autosomal X-shredder becoming a driving-Y chromosome and invasively suppressing wild populations of malaria mosquitoes. *Frontiers in Bioengineering and Biotechnology* 9, 752253. doi: 10.3389/fbioe.2021.752253

Alphey, L. (2014) Genetic control of mosquitoes. *Annual Review of Entomology* 59, 205–224. doi: 10.1146/annurev-ento-011613-162002

Alphey, L., Benedict, M., Bellini, R., Clark, G.G., Dame, D.A. *et al.* (2010) Sterile-insect methods for control of mosquito-borne diseases: an analysis. *Vector-Borne and Zoonotic Diseases* 10, 295–311. doi: 10.1089/vbz.2009.0014

Andreasen, M.H., and Curtis, C.F. (2005) Optimal life stage for radiation sterilization of Anopheles males and their fitness for release. *Medical and Veterinary Entomology* 19, 238–244.

Aryan, A., Anderson, M.A.E., Biedler, J.K., Qi, Y., Overcash, J.M. *et al.* (2020) Alone is sufficient to convert female into fertile males and is needed for male flight. *Proceedings of the National Academy of Sciences USA* 117, 17702–17709.

Bachtrog, D. (2013) Y-chromosome evolution: emerging insights into processes of Y-chromosome degeneration. *Nature Reviews. Genetics* 14, 113–124.

Baker, R.H. and Sakai, R.K. (1979) Triploids and male determination in the mosquito, *Anopheles culicifacies*. *The Journal of Heredity* 70, 345–346.

Balatsos, G., Puggioli, A., Karras, V., Lytra, I., Mastronikolos, G. *et al.* (2021) Reduction in egg fertility of mosquitoes in Greece following releases of imported sterile males. *Insects* 12, 110. doi: 10.3390/insects12020110

Bayega, A., Djambazian, H., Tsoumani, K.T., Gregoriou, M.E., Sagri, E. *et al.* (2020) De novo assembly of the olive fruit fly (*Bactrocera oleae*) genome with linked-reads and long-read technologies minimizes gaps and provides exceptional Y chromosome assembly. *BMC Genomics* 21, 259. doi: 10.1186/s12864-020-6672-3

Bell, L.R., Horabin, J.I., Schedl, P. and Cline, T.W. (1991) Positive autoregulation of sex-lethal by alternative splicing maintains the female determined state in Drosophila. *Cell* 65, 229–239. doi: 10.1016/0092-8674(91)90157-t

Bellini, R., Medici, A., Puggioli, A., Balestrino, F. and Carrieri, M. (2013a) Pilot field trials with *Aedes albopictus* irradiated sterile males in Italian urban areas. *Journal of Medical Entomology* 50, 317–325.

Bellini, R., Balestrino, F., Medici, A., Gentile, G., Veronesi, R. and M. Carrieri, M. (2013b) Mating competitiveness of *Aedes albopictus* radio-sterilized males in large enclosures exposed to natural conditions. *Journal of Medical Entomology* 50, 94–102.

Bernardini, F., Galizi, R., Menichelli, M., Papathanos, P.-A., Dritsou, V. *et al.* (2014) Site-specific genetic engineering of the *Anopheles gambiae* Y chromosome. *Proceedings of the National Academy of Sciences USA* 111, 7600–7605.

Beukeboom, L.W. and van de Zande, L. (2010) Genetics of sex determination in the haplodiploid wasp *Nasonia vitripennis* (Hymenoptera: Chalcidoidea). *Journal of Genetics* 89, 333–339.

Bonaccorsi, S., Gatti, M., Pisano, C. and Lohe, A. (1990) Transcription of a satellite DNA on two Y chromosome loops of *Drosophila melanogaster*. *Chromosoma* 99, 260–266.

Bracewell, R., Tran, A., Chatla, K. and Bachtrog, D. (2020) Chromosome-level assembly of *Drosophila bifasciata* reveals important karyotypic transition of the X chromosome. *G3 (Genes, Genomes, Genetics)* 10, 891–897. doi: 10.1101/847558.

Brand, C.L., Larracuente, A.M. and Presgraves, D.C. (2015) Origin, evolution, and population genetics of the selfish segregation distorter gene duplication in European and African populations of *Drosophila melanogaster*. *Evolution (International Journal of Organic Evolution)* 69, 1271–1283.

Buchman, A., Shriner, I., Yang, T., Liu, J., Igor Antoshechkin, I. *et al.* (2021) Engineered reproductively isolated species drive reversible population replacement. *Nature Communications* 12, 3281.

Burt, A. (2003) Site-specific selfish genes as tools for the control and genetic engineering of natural populations. *Proceedings of the Royal Society B: Biological Sciences* 270 (1518), 921–928.

Burt, A. and Deredec, A. (2018) Self-limiting population genetic control with sex-linked genome editors. *Proceedings of the Royal Society B: Biological Sciences* 285 (1883), 20180776. doi: 10.1098/rspb.2018.0776

Bushland, R.C., Lindquist, A.W. and Knipling, E.F. (1955) Eradication of screw-worms through release of sterilized males. *Science* 122 (3163), 287–88.

Carrami, E.M., Eckermann, K.N., Ahmed, H.M.M., Sánchez Castellanos, H.M., Dippel, S. *et al.* (2018) Consequences of resistance evolution in a Cas9-based sex conversion-suppression gene drive for insect pest management. *Proceedings of the National Academy of Sciences USA* 115, 6189–6194. doi: 10.1073/pnas.1713825115

Carvalho, A.B., Vicoso, B., Russo, C.A.M., Swenor, B. and Clark, A.G. (2015a) Birth of a new gene on the Y chromosome of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences USA* 112, 12450–12455.

Carvalho, D.O., McKemey, A.R., Garziera, L., Lacroix, R., Donnelly, C.A. *et al.* (2015b) Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. *PLoS Neglected Tropical Diseases* 9, e0003864. doi: 10.1371/journal.pntd.0003864.

Catteruccia, F., Benton, J.P. and Crisanti, A. (2005) An *Anopheles* transgenic sexing strain for vector control. *Nature Biotechnology* 23, 1414–1417.

Cha, S.-J., Chadee, D.D. and Severson, D.W. (2006) Population dynamics of an endogenous meiotic drive system in *Aedes aegypti* in Trinidad. *The American Journal of Tropical Medicine and Hygiene* 75, 70–77.

Cho, S., Huang, Z.Y. and Zhang, J. (2007) Sex-specific splicing of the honeybee doublesex gene reveals 300 million years of evolution at the bottom of the insect sex-determination pathway. *Genetics* 177, 1733–1741. doi: 10.1534/genetics.107.078980

Cline, T.W. (1984) Autoregulatory functioning of a *Drosophila* gene product that establishes and maintains the sexually determined state. *Genetics* 107, 231–277. doi: 10.1093/genetics/107.2.231

Cline, T.W. and Meyer, B.J. (1996) Vive la différence: males vs females in flies vs worms. *Annual Review of Genetics* 30, 637–702.

Collins, F.H., Mendez, M.A. Rasmussen, M.O. Mehaffey P.C., Besansky, N.J. and Finnerty, V. (1987) A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. *The American Journal of Tropical Medicine and Hygiene* 37, 37–41.

Collins, F.H., Paskewitz, S.M. and Finnerty, V. (1989) Ribosomal RNA genes of the *Anopheles gambiae* species complex. In: Harris, K.F. (ed.) *Advances in Disease Vector Research*, Vol. 6. Springer Verlag, New York, pp. 1–28. doi: https://doi.org/10.1007/978-1-4612-3292-6_1.

Concha, C. and Scott, M.J. (2009) Sexual development in *Lucilla cuprina* (Diptera, Calliphoidae) is controlled by the transformer gene. *Genetics* 162(3), 785–798.

Craig, G.B. Jr and Hickey, W.A. (1967) Current status of the formal genetics of *Aedes aegypti*. *Bulletin of the World Health Organization* 36, 559–562.

Criscione, F., Qi, Y., Saunders, R., Hall, B. and Tu, Z. (2013) A unique Y gene in the Asian malaria mosquito *Anopheles stephensi* encodes a small lysine-rich protein and is transcribed at the onset of embryonic development. *Insect Molecular Biology* 22, 433–441. doi: 10.1111/imb.12034

Criscione, F., Qi, Y. and Tu, Z. (2016) *GUY1 confers complete female lethality and is a strong candidate for a male-determining factor in Anopheles stephensi. eLife* 5, e19281. doi: 10.7554/eLife.19281.

Curtis, C.F. (1985) Genetic control of insect pests: growth industry or lead balloon? *Biological Journal of the Linnean Society* 26, 359–374.

Dietz, K. (1976) The effect of immigration on genetic control. *Theoretical Population Biology* 9, 58–67. doi: 10.1016/0040-5809(76)90035-6

Erickson, J.W. and Quintero, J.J. (2007) Indirect effects of ploidy suggest X chromosome dose, not the X:A ratio, signals sex in *Drosophila*. *PLoS Biology* 5, e332.

Fasulo, B., Meccariello, A., Morgan, M., Borufka, C., Papathanos, P.A. and Windbichler, N. (2020) A fly model establishes distinct mechanisms for synthetic CRISPR/Cas9 sex distorters. *PLoS Genetics* 16, e1008647.

Galizi, R., Doyle, L.A., Menichelli, M., Bernardini, F., Deredec, A. *et al.* (2014) A synthetic sex ratio distortion system for the control of the human malaria mosquito. *Nature Communications* 5, 3977.

Galizi, R., Hammond, A., Kyrou, K., Taxiarchi, C., Bernardini, F. *et al.* (2016) A CRISPR-Cas9 sex-ratio distortion system for genetic control. *Scientific Reports* 6, 31139.

Gould, F. and Schliekelman, P. (2004) Population genetics of autocidal control and strain replacement. *Annual Review of Entomology* 49, 193–217.

Hackstein, J.H. and Hochstenbach, R. (1995) The elusive fertility genes of *Drosophila*: the ultimate haven for selfish genetic elements. *Trends in Genetics* 11, 195–200.

Hall, A.B., Qi, Y., Timoshevskiy, V., Sharakhova, M.V., Sharakhov, I.V. and Tu, Z. (2013) Six novel Y chromosome genes in *Anopheles* mosquitoes discovered by independently sequencing males and females. *BMC Genomics* 14, 273. doi: 10.1186/1471-2164-14-273

Hall, A.B., Basu, S., Jiang, S., Qi, Y., Timoshevskiy, V.A. *et al.* (2015) sex determination. a male-determining factor in the mosquito *Aedes aegypti*. *Science* 348, 1268–1270.

Hall, A.B., Papathanos, P.-A., Sharma, A., Cheng, C., Akbari, O.S. *et al.* (2016) Radical remodeling of the Y chromosome in a recent radiation of malaria mosquitoes. *Proceedings of the National Academy of Sciences USA* 113, E2114–E2123.

Hamilton, W.D. (1967) Extraordinary sex ratios. A sex-ratio theory for sex linkage and inbreeding has new implications in cytogenetics and entomology. *Science* 156, 477–488.

Hammer, M.F. (1991) Molecular and chromosomal studies on the origin of T haplotypes in mice. *The American Naturalist* 137, 135–165.

Hammond, A.M., Kyrou, K., Bruttini, M., North, A., Galizi, R. *et al.* (2017) The creation and selection of mutations resistant to a gene drive over multiple generations in the malaria mosquito. *PLoS Genetics* 13, e1007039.

Harris, A.F., Nimmo, D., McKemey, A.R., Kelly, N., Scaife, S. *et al.* (2011) Field performance of engineered male mosquitoes. *Nature Biotechnology* 29, 1034–1037.

Harris, A.F., McKemey, A.R., Nimmo, D., Curtis, Z., Black, I. *et al.* (2012) Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nature Biotechnology* 30, 828–830.

Hediger, M., Burghardt, G., Siegenthaler, C., Buser, N., Hilfiker-Kleiner, D. *et al.* (2004) Sex determination in *Drosophila melanogaster* and *Musca domestica* converges at the level of the terminal regulator doublesex. *Development Genes and Evolution* 214, 29–42. doi: 10.1007/s00427-003-0372-2

Hickey, W.A., and Craig, G.B. Jr (1966) Distortion of sex ratio in populations of *Aedes aegypti*. *Canadian Journal of Genetics and Cytology (Journal canadien de génétique et de cytologie)* 8, 260–278.

Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R. *et al.* (2002) The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science* 298, 129–149.

Hughes, J.F. and Page, D.C. (2015) The biology and evolution of mammalian Y Chromosomes. *Annual Review of Genetics* 49, 507–527.

Inoue, H. and Hiroyoshi, T. (1986) A maternal-effect sex-transformation mutant of the housefly, *Musca domestica* L. *Genetics* 112, 469–482.

Kaiser, V.B. and Bachtrog, D. (2010) Evolution of sex chromosomes in insects. *Annual Review of Genetics* 44, 91–112.

Kiuchi, T., Koga, H., Kawamoto, M., Shoji, K., Sakai, H. *et al.* (2014) A single female-specific piRNA is the primary determiner of sex in the silkworm. *Nature* 509, 633–636.

Klein, T.A., Windbichler, N., Deredec, A., Burt, A. and Benedict, M.Q. (2012) Infertility resulting from transgenic I-PpoI male *Anopheles gambiae* in large cage trials. *Pathogens and Global Health* 106(1), 20–31. doi: 10.1179/2047773212Y.0000000003

Knipling, E.F. (1955) Possibilities of insect control or eradication through the use of sexually sterile males. *Journal of Economic Entomology* 48, 459–462. doi: 10.1093/jee/48.4.459

Kozielska, M., Feldmeyer, B., Pen, I., Weissing, F.J. and Beukeboom, L.W. (2008) Are autosomal sex-determining factors of the housefly (*Musca domestica*) spreading north? *Genetics Research* 90, 157–165.

Krafsur, E.S., Townson, H., Davidson, G. and Curtis, C.F. (1986) Screwworm eradication is what it seems. *Nature* 323, 495–496.

Krafsur, E.S., Whitten, C.J. and Novy, J.E. (1987) Screwworm eradication in North and Central America. *Parasitology Today* 3, 131–137.

Krzywinska, E. and Krzywinski, J. (2018) Effects of stable ectopic expression of the primary sex determination gene *yob* in the mosquito *Anopheles gambiae*. *Parasites & Vectors* 11 (Suppl. 2), 648.

Krzywinska, E., Dennison, N.J., Lycett, G.L. and Krzywinski, J. (2016) A maleness gene in the malaria mosquito *Anopheles gambiae*. *Science* 353, 67–69.

Krzywinska, E., Ferretti, L., Li, J., Li, J.-C., Chen, C.-H. and Krzywinski, J. (2021) Femaleless controls sex determination and dosage compensation pathways in females of *Anopheles* mosquitoes. *Current Biology* 31, 1084–1091.e4.

Krzywinski, J., Nusskern, D.R., Kern, M.K. and Besansky, N.J. (2004) Isolation and characterization of Y chromosome sequences from the African malaria mosquito *Anopheles gambiae*. *Genetics* 166, 1291–1302.

Kyrou, K., Hammond, A.M., Galizi, R., Kranjc, N., Burt, A. *et al.* (2018) A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged *Anopheles gambiae* mosquitoes. *Nature Biotechnology* 36, 1062–1066.

Labbé, G.M.C., Scaife, S., Morgan, S.A., Curtis, Z.H. and Alphey, L. (2012) Female-specific flightless (fsRIDL) phenotype for control of *Aedes albopictus*. *PLoS Neglected Tropical Diseases* 6, e1724.

Lacroix, R., McKemey, A.R., Raduan, N., Wee, L.K., Ming, W.H. *et al.* (2012) Open field release of genetically engineered sterile male *Aedes aegypti* in Malaysia. *PLoS ONE* 7, e42771.

Lagos, D., Koukidou, M., Savakis, C. and Komitopoulou, K. (2007) The transformer gene in *Bactrocera oleae*: the genetic switch that determines its sex fate. *Insect Molecular Biology* 16, 221–230.

Larson, E.L., Kopania, E.E.K. and Good, J.M. (2018) Spermatogenesis and the evolution of mammalian sex chromosomes. *Trends in Genetics (TIG)* 34, 722–732.

Lutrat, C., Olmo, R.P., Baldet, T., Bouyer, J. and Marois, E. (2022) Transgenic expression of *Nix* converts genetic females into males and allows automated sex sorting in *Aedes albopictus*. *Communications Biology* 5, 10. doi: 10.1038/s42003-022-03165-7

Lyttle, T.W. (1991) Segregation distorters. *Annual Review of Genetics* 25, 511–557.

Magnusson, K., Lycett, G.J., Mendes, A.M., Lynd, A., Papathanos, P.-A. *et al.* (2012) Demasculinization of the *Anopheles gambiae* X chromosome. *BMC Evolutionary Biology* 12, 69.

Mahajan, S., Wei, K., Nalley, M., Gibilisco, L. and Bachtrog, D. (2018) De novo assembly of a young *Drosophila* Y chromosome using single-molecule sequencing and chromatin conformation capture. *PLoS Biology* 16, e2006348.

McKee, B.D. and Handel, M.A. (1993) Sex chromosomes, recombination, and chromatin conformation. *Chromosoma* 102, 71–80.

Meccariello, A., Krsticevic, F., Colonna, R., Del Corsano, G., Fasulo, B. *et al.* (2021) Engineered sex ratio distortion by X-shredding in the global agricultural pest *Ceratitis capitata*. *BMC Biology* 19, 78.

Newton, M.E., Southern, D.I. and Wood, R.J. (1974) X and Y chromosomes of *Aedes aegypti* (L.) distinguished by giemsa C-banding. *Chromosoma* 49, 41–49.

Ohbayashi, F., Suzuki, M.G., Mita, K., Okano, K. and Shimada, T. (2001) A homologue of the *Drosophila* doublesex gene is transcribed into sex-specific mRNA isoforms in the silkworm, *Bombyx mori*. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology* 128, 145–158.

O'Leary, S. and Adelman, Z.N. (2020) CRISPR/Cas9 knockout of female-biased genes AeAct-4 or Myo-Fem in *Ae. aegypti* results in a flightless phenotype in female, but not male mosquitoes. *PLoS Neglected Tropical Diseases* 14, e0008971.

Pane, A., Salvemini, M., Delli Bovi, P., Polito, C. and Saccone, G. (2002) The transformer gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. *Development* 129, 3715–3725.

Papa, F., Windbichler, N., Waterhouse, R.M., Cagnetti, A., D'Amato, R. *et al.* (2017) Rapid evolution of female-biased genes among four species of *Anopheles* malaria mosquitoes. *Genome Research* 27, 1536–1548. doi: 10.1101/gr.217216.116

Papathanos, P.A. and Windbichler, N. (2018) Redkmer: an assembly-free pipeline for the identification of abundant and specific X-chromosome target sequences for X-shredding by CRISPR endonucleases. *The CRISPR Journal* 1, 88–98.

Paskewitz, S.M. and Collins, F.H. (1990) Use of the polymerase chain reaction to identify mosquito species of the *Anopheles gambiae* complex. *Medical and Veterinary Entomology* 4, 367–373. doi: 10.1111/j.1365-2915.1990.tb00453.x

Pertile, M.D., Graham, A.N., Choo, K.H.A. and Kalitsis, P. (2009) Rapid evolution of mouse Y centromere repeat DNA belies recent sequence stability. *Genome Research* 19, 2202–2213.

Pollegioni, P., North, A.R., Persampieri, T., Bucci, A., Minuz, R.L. *et al.* (2020) Detecting the population dynamics of an autosomal sex ratio distorter transgene in malaria vector mosquitoes. *The Journal of Applied Ecology* 57, 2086–2096.

Pomiankowski, A. (2004) The evolution of the drosophila sex-determination pathway. *Genetics* 166, 1761–1773. doi: 10.1534/genetics.166.4.1761

Prout, T. (1978) The joint effects of the release of sterile males and immigration of fertilized females on a density regulated population. *Theoretical Population Biology* 13, 40–71.

Qi, Y., Wu, Y., Saunders, R., Chen, X.-G., Mao, C. *et al.* (2019) *Guy1, a Y-linked embryonic signal, regulates dosage compensation in Anopheles stephensi by increasing X gene expression. eLife* 8, e43570. doi: 10.7554/eLife.43570.

Robinson, A.S. (1983) Sex-ratio manipulation in relation to insect pest control. *Annual Review of Genetics* 17, 191–214.

Rose, G., Krzywinska, E., Kim, J., Revuelta, L., Ferretti, L. and Krzywinski, J. (2016) Dosage compensation in the African malaria mosquito *Anopheles gambiae*. *Genome Biology and Evolution* 8, 411–425. doi: 10.1093/gbe/evw004

Sakai, R.K. and Miller, L.H. (1992) Effects of heat shock on the survival of transgenic *Anopheles gambiae* (Diptera: Culicidae) under antibiotic selection. *Journal of Medical Entomology* 29, 374–375.

Salvemini, M., Mauro, U., Fabrizio Lombardo, F., Milano, A., Zazzaro, V. *et al.* (2011) Genomic organization and splicing evolution of the doublesex gene, a *Drosophila* regulator of sexual differentiation, in the dengue and yellow fever mosquito *Aedes aegypti*. *BMC Evolutionary Biology* 11, 41.

Sarno, F., Ruiz, M.F., Eirín-López, J.M., Perondini, A.L.P., Selivon, D and Sánchez, L. (2010) The gene transformer-2 of *Anastrepha* fruit flies (Diptera, Tephritidae) and its evolution in insects. *BMC Evolutionary Biology* 10, 140.

Schetelig, M.F., Milano, A., Saccone, G. and Handler, A.M. (2012) Male only progeny in *Anastrepha suspensa* by RNAi-induced sex reversion of chromosomal females. *Insect Biochemistry and Molecular Biology* 42, 51–57.

Schliekelman, P., Ellner, S. and Gould, F. (2005) Pest control by genetic manipulation of sex ratio. *Journal of Economic Entomology* 98, 18–34.

Scolari, F., Schetelig, M.F., Bertin, S., Malacrida, A.R., Gasperi, G. and Wimmer, E.A. (2008) Fluorescent sperm marking to improve the fight against the pest insect *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae). *New Biotechnology* 25, 76–84.

Scott, M. (2021) Sex determination and dosage compensation: *femaleless* is the link in *Anopheles* mosquitoes. *Current Biology* 31, R260–R263.

Shearman, D.C.A. (2002) The evolution of sex determination systems in dipteran insects other than *Drosophila*. *Genetica* 116, 25–43.

Shukla, J.N. and Nagaraju, J. (2010) Doublesex: a conserved downstream gene controlled by diverse upstream regulators. *Journal of Genetics* 89, 341–356.

Shukla, J.N., Jadhav, S. and Nagaraju, J. (2011) Novel female-specific splice form of dsx in the silkworm, *Bombyx mori*. *Genetica* 139, 23–31.

Simoni, A., Hammond, A.M., Beaghton, A.K., Galizi, R., Taxiarchi, C. *et al.* (2020) A male-biased sex-distorter gene drive for the human malaria vector *Anopheles gambiae*. *Nature Biotechnology* 38, 1054–1060.

Smith, R.C., Walter, M.F., Hice, R.H., O'Brochta, D.A. and Atkinson, P.W. (2007) Testis-specific expression of the beta2 tubulin promoter of *Aedes aegypti* and its application as a genetic sex-separation marker. *Insect Molecular Biology* 16, 61–71.

Sweeny, T.L. and Barr, A.R. (1978) Sex ratio distortion caused by meiotic drive in a mosquito, *Culex pipiens* L. *Genetics* 88, 427–446.

Taxiarchi, C., Kranjc, N., Kriezis, A., Kyrou, K., Bernardini, F. *et al.* (2019) High-resolution transcriptional profiling of *Anopheles gambiae* spermatogenesis reveals mechanisms of sex chromosome regulation. *Scientific Reports* 9, 14841.

Vibranovski, MD. (2014) Meiotic sex chromosome inactivation in *Drosophila*. *Journal of Genomics* 2, 104–117.

Vibranovski, M.D., Zhang, Y.E., Kemkemer, C., Lopes, H.F., Karr, T.L. and Long, M. (2012) Re-analysis of the larval testis data on meiotic sex chromosome inactivation revealed evidence for tissue-specific gene expression related to the *Drosophila* X chromosome. *BMC Biology* 10, 49.

Vicoso, B. and Bachtrog, D. (2015) Numerous transitions of sex chromosomes in Diptera. *PLoS Biology* 13, e1002078.

Waltz, E. (2021) First genetically modified mosquitoes released in the United States. *Nature* 593, 175–176.

Ward, C.M., Aumann, R.A., Whitehead, M.A., Nikolouli, K., Leveque, G. *et al.* (2021) White pupae phenotype of Tephritids is caused by parallel mutations of a MFS transporter. *Nature Communications* 12, 491.

Whitten, M.J. (1985) The conceptual basis for genetic control. In: Kerkut, G.A. and Gilbert, L.I. *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*. Pergamon Press, Oxford, pp. 465–527.

Wilkins, A.S. (1995) Moving up the hierarchy: a hypothesis on the evolution of a genetic sex determination pathway. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology* 17, 71–77.

Willhoeft, U. and Franz, G. (1996) Identification of the sex-determining region of the *Ceratitis capitata* Y chromosome by deletion mapping. *Genetics* 144, 737–745.

Windbichler, N., Papathanos, P.A. and Crisanti, A. (2008) Targeting the X chromosome during spermatogenesis induces Y chromosome transmission ratio distortion and early dominant embryo lethality in *Anopheles gambiae*. *PLoS Genetics* 4, e1000291.

Wood, R.J. and Newton, M.E. (1991) Sex-ratio distortion caused by meiotic drive in mosquitoes. *The American Naturalist* 137, 379–391. doi: 10.1086/285171

Zhang, D., Lees, R.S., Xi, Z., Gilles, J.R.L. and Bourtzis, K. (2015) Combining the sterile insect technique with *Wolbachia*-based approaches: II- A safer approach to *Aedes albopictus* population suppression programmes, designed to minimize the consequences of inadvertent female release. *PLoS ONE* 10, e0135194. doi: 10.1371/journal.pone.0135194

Zheng, X., Zhang, D., Li, Y., Yang, C., Wu, Y. *et al.* (2019) Incompatible and sterile insect techniques combined eliminate mosquitoes. *Nature* 572, 56–61.

Zimmering, S., Sandler, L. and Nicoletti, B. (1970) Mechanisms of meiotic drive. *Annual Review of Genetics* 4, 409–436. doi: 10.1146/annurev.ge.04.120170.002205

Zimowska, G.J., Nirmala, X. and Handler, A.M. (2009) The beta2-tubulin gene from three tephritid fruit fly species and use of its promoter for sperm marking. *Insect Biochemistry and Molecular Biology* 39, 508–515.

11 Population Modification Using Gene Drive for Reduction of Malaria Transmission

Vanessa Bottino-Rojas[1] and Anthony A. James[1,2]*

[1]*Department of Microbiology and Molecular Genetics, University of California, Irvine, CA, USA;* [2]*Department of Molecular Biology and Biochemistry, University of California, Irvine, CA, USA*

11.1 Introduction

The global incidence of malaria has been reduced in the past 15 years and much of this success results from the application of vector control methods to prevent pathogen transmission (Bhatt *et al*., 2015). However, the decline in incidence has slowed recently and disease incidence and prevalence remain high (World Health Organization, 2020). Traditional vector control methods, although effective in many regional and local applications, are limited in others and may be complemented by new synthetic biological tools. Transgenic and genome-edited mosquitoes can carry special traits for spread into wild populations that are expected to assist in local elimination and contribute ultimately to disease eradication (Carballar-Lejarazú and James, 2017). These traits can be designed to reduce the ability of vector mosquitoes to sustain parasite transmission, or to decrease their lifespan or ability to reproduce, known respectively as population alteration/modification/replacement (henceforth referred to as 'population modification') and population suppression.

The prospect of driving genetic modifications that confer malaria-parasite refractory or resistance qualities into mosquito vectors has been captivating scientists for more than 50 years (Curtis, 1968). Naturally occurring 'selfish' genetic elements were proposed as a way to achieve genetically engineered populations (Curtis, 1992; Burt, 2003). However, the major technological breakthrough in the field came only recently with the discovery of CRISPR/Cas9-based gene editing approaches, which offer simplicity and efficiency when compared with other genome editing tools (Ran *et al*., 2013; see Concha and Papa, Chapter 7, this volume). Since then, studies of vector–pathogen biology and malaria immunology combined with the development of molecular tools for manipulating mosquito genomes have been fuelling new developments, including mosquitoes that express endogenous (originating within the mosquito) or exogenous (derived from external sources) anti-parasite effectors, or lack important host factors that affect *Plasmodium* transmission (Sreenivasamurthy *et al*., 2013; Carballar-Lejarazú and James, 2017; Simões *et al*., 2018).

We discuss here the application of some of these studies and how vector manipulation tools such as those derived from CRISPR/Cas9-based gene drive technologies can be

*Corresponding author, email: aajames@uci.edu

used to modify anopheline mosquito populations. We examine the core concepts and mechanisms for inducing efficient modifications that have already been identified or that represent promising targets. In the final section, we discuss how effective these transmission-blocking interventions must be to adequately support malaria elimination efforts.

11.2 Features of Gene Drive Population Modification Systems

Gene drive systems in mosquitoes utilize CRISPR/Cas9-based technology in which an endonuclease, Cas9, makes a double-strand break in the chromosomal DNA at a specific 'target' site directed by a guide RNA (gRNA)

(Fig. 11.1). Subsequent homology-directed repair and recombination can then be used to insert specific exogenous DNA into the cleavage site. If this exogenous DNA contains the Cas9 endonuclease and gRNA, a self-propagating (autonomous) genetic element will continue to cut-and-paste itself into any appropriate target site, thus generating gene drive (see Raban and Akbari, Chapter 8; Champer, Chapter 9, this volume). Non-autonomous systems also can be developed by unlinking the Cas9-encoding gene from the gRNA (Gantz and Bier, 2015). Comprehensive information on insect germline transformation and overall CRISPR/Cas9 knock-in methods are described elsewhere in this book (see O'Brochta, Chapter 1; Ahmed and Wimmer, Chapter 5, this volume).

A number of practical considerations guide the design and construction of population
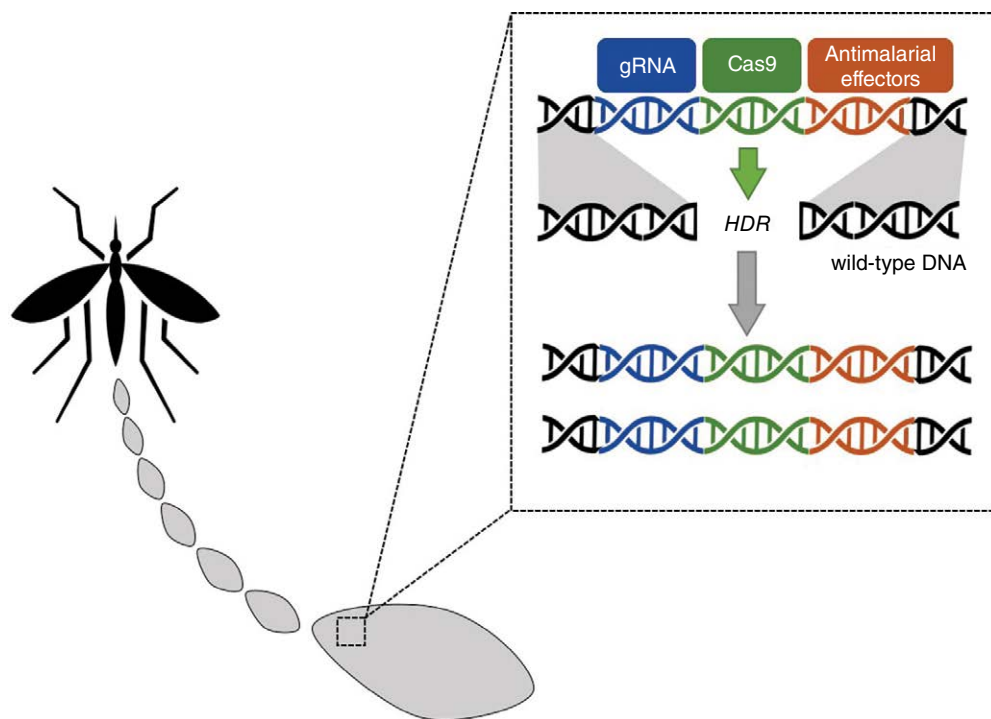
Fig. 11.1. Schematic representation of Cas9/gRNA-mediated gene drive. A gene drive element, including the Cas9, a guide RNA (gRNA) targeting the gene of choice and the antimalarial molecules, is inserted at the homologous chromosomal Cas9-cleaved locus, through DNA repair by homologous recombination (HDR). The occurrence of this event in the germline favours the inheritance of the transgenic cassette. The ability to maintain inheritance of transgenically encoded traits despite potential fitness defects enables driving of otherwise costly beneficial genetic traits through wild populations.

modification gene drive systems (Carballar-Lejarazú and James, 2017). For example, it is important that the components of a synthetic construct be as short in nucleotide length as possible and still allow appropriate function. The smaller size of components reduces the probability of adventitious sequence similarity to other components of the system or the target genome.

Furthermore, the likelihood of generating an unintended recombination event depends on the activity, specificity, dose and spatio-temporal regulation of the nuclease, the sequence of its target and flanking region, the presence and frequency of genomic sequences resembling the target, and the accessibility of these sites to the nuclease and DNA repair machinery. In the first attempt to engineer a synthetic gene drive system using custom zinc-finger and transcription activator-like effector nucleases (TALEN) in *Drosophila melanogaster*, the repetitive sequences of the nuclease genes led to deletion and rearrangement events that decreased the drive efficiency (Simoni *et al.*, 2014). In mosquitoes, rare (about 1×10^{-5}) insertions and deletions recovered during small cage trials were attributed to recombination between small (about 20 nucleotides) direct repeat sequences in the genomic target site and drive system DNA (Pham *et al.*, 2019). It is currently accepted that the use of an improved Cas9–gRNA element design lacking direct repeat sequences reduces the probability of disruption in the drive system as well the generation of mutations or rearrangement of the host genome.

The locus of the target site specified by the gRNA in the mosquito genome should be well characterized and conserved across populations of the same species. Characterization includes screening mosquito genomes for naturally occurring target-site polymorphisms that may halt or reduce drive dynamics. However, this is a challenge, because highly conserved target sites may be so because they are under negative selection and the insertion of a large gene drive element into them may have a deleterious effect. While this may be a benefit to suppression approaches, a well-designed population modification system will avoid this.

In addition to naturally occurring variants in the target site, drive-resistant alleles may be generated by non-homologous end joining (NHEJ) events during drive. As targets that are highly conserved in nature are indicative of strong sequence constraints, an NHEJ resistance allele that still encodes a functional product may be generated misrepair and increase in frequency (Hammond and Galizi, 2017).

Insertion of exogenous DNA at a target site is potentially mutagenic for the gene in which the site is located and this could impose a genetic load as a result of the interrupted gene (insertion-site effect) or expression of the inserted DNA (transgene effect). These alterations could make the resulting mosquitoes less competitive with wild-type counterparts.

An example of informed decision making during target selection is presented by Carballar-Lejarazú *et al.* (2020) in the development of an efficient gene drive system in the African malaria vector, *Anopheles gambiae*. Through target-site sequence screening of genomes of natural populations and Cas9/gRNA *in vitro* cleavage assays of polymorphic alleles with potentially critical modifications in the sequence, the authors concluded that naturally prevalent or NHEJ-induced resistant alleles are not likely to prevent (only delay) delivery of the intended modification (Carballar-Lejarazú *et al.*, 2020). Furthermore, resistant alleles are not expected to rise in frequency if the targeted sequences cannot tolerate disruption (Unckless *et al.*, 2017). Therefore, an alternative strategy uses a gRNA sequence that purposely targets an essential gene and links the anti-malarial effector and drive cassette to a sequence that restores the essential function and is resistant to cutting (Beaghton *et al.*, 2017; Noble *et al.*, 2017). This was demonstrated successfully on a recoded gene drive rescue system for population modification of the Indo-Pakistan malaria vector, *An. stephensi*; individuals carrying NHEJ alleles had a low fitness and were purged quickly from the population (Adolfi *et al.*, 2020).

The rationale for target site selection must be appropriate for the proposed modification strategy. The intended modification

might require disruption of a predetermined target (for example, insecticide-resistant gene or host-factor for parasite development) or an insertion of a novel anti-pathogen effector. As mentioned, there is a trade-off between the targeting of a conserved sequence with a potential resulting fitness cost versus the insertion of the transmission-blocking gene on a neutral genomic site. The latter usually exhibits poor sequence conservation and facilitates the emergence of drive resistance, because non-functional regions can more easily accommodate cost-free sequence alterations, especially if the gene drive allele carries a fitness cost.

Another concern for modification systems is that mutations can occur in the effector genes, rendering them non-functional and leaving a drive-only cargo to be spread through a population, with no effect on disease transmission. This may be challenging to measure, considering that the spread of this non-functional replacement could be much slower than that of the initial drive system and fixation of the effector-drive construct. Although the technology performance in the field may be different from that in the laboratory, parameters such as fitness and vector competence of the modified mosquitoes should be tested through multiple generations with appropriate laboratory cage trials (Pham *et al.*, 2019). Furthermore, drive experiments on recently colonized local populations and field trials in naturally isolated settings in the wild are relevant to test laboratory predictions and contemplate long-term population dynamics and persistence of the introduced modification (Carballar-Lejarazú and James, 2017; Schmidt *et al.*, 2020). Mathematical modelling can be used to explore and assess the utility of a wide range of scenarios that would be costly, time-consuming or even unfeasible to test experimentally (Robert *et al.*, 2012; see Edgington and Alphey, Chapter 12, this volume). Despite the fact that effector or drive loss-of-function mutations may arise beyond our testing abilities, disease protection may nonetheless persist sufficiently long enough to provide a public health benefit (Beaghton *et al.*, 2017).

11.3   Design Features of Parasite-Resistant Mosquitoes for Population Modification

Improved understanding of mosquito–pathogen interactions and developments of immune-related or synthetic-derived anti-malarial factors have rapidly advanced prospects for generating refractory mosquito populations (Caragata *et al.*, 2020). Similarly, progress in mosquito genomics has enabled the design and production of engineered genes expressed under the control of specific promoter-regulatory DNA, achieving great parasite blocking performances in laboratory settings, mostly through transposon-based transgenesis (Chen *et al.*, 2008; Häcker and Schetelig, 2018). Reviewing the landmarks that built the current knowledge and achievements can enlighten prospective and innovative ideas for future implementation of this technology.

Population-wide genetic modifications benefit if the transformed insects exhibit as low a fitness cost as possible. Therefore, the expression of effector genes should be restricted ideally to infection-relevant development phases and tissues in the mosquito. To complete their transmission cycle, *Plasmodium* parasites must traverse three key mosquito compartments, the midgut, the haemocoel and the salivary glands, during the course of several days after initial acquisition by the mosquito through an infectious bloodmeal (Fig. 11.2). Different *cis*-acting control DNA sequences that regulate appropriate patterns of expression have been identified in malaria vectors.

Mosquito midgut invasion is a crucial step to parasite infection, as this is the first host tissue encountered by the parasites. Therefore, control DNA sequences, particularly those that are bloodmeal-inducible and regulate midgut-specific expression, confer abundant activation of the effector genes that is synchronized with parasite ingestion (see Nolan and Hammond, Chapter 3, this volume). Examples of such control sequences come from genes that encode a zinc carboxypeptidase A1 (*CP*), a late trypsin (*Antryp1*) and the *G12* regulatory elements
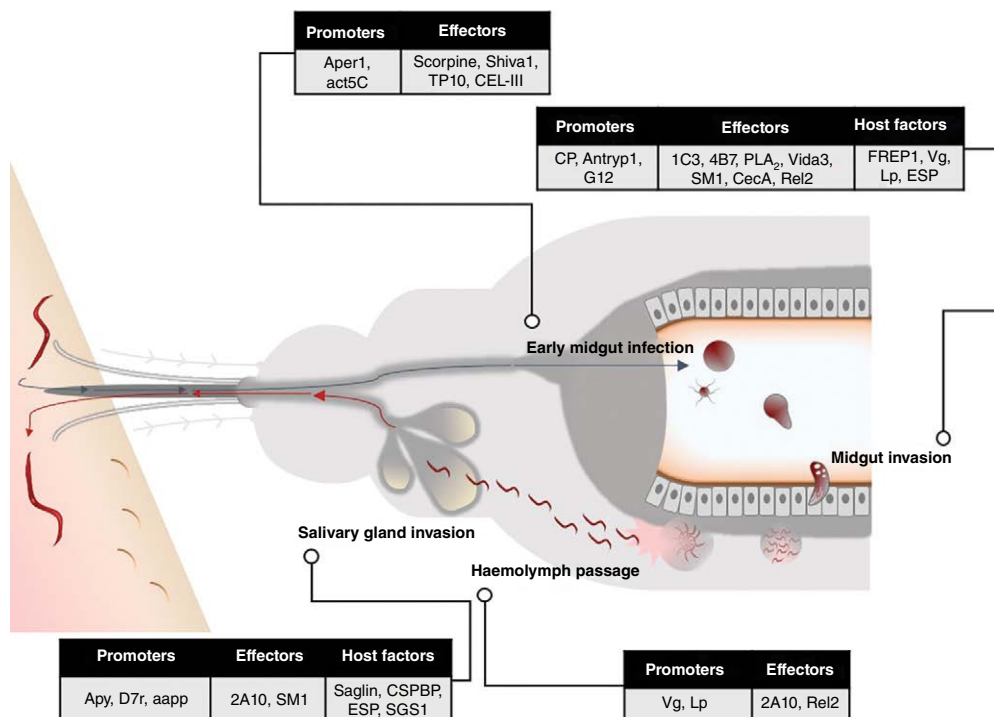
Fig. 11.2. Genetic targets for modification during malaria parasite development in the mosquito host. Plasmodium parasites enter the mosquito midgut lumen through a bloodmeal. The sporogonic cycle starts with the gametocytes' maturation into female and male gametes (early midgut infection). Upon fertilization, the zygote is formed and develops into an ookinete. Ookinetes cross the peritrophic matrix and traverse the midgut epithelium (midgut invasion) before settling in its basal side, where they develop into oocysts. Oocyst maturation results in the production of thousands of sporozoites that are released into the haemocoel (haemolymph passage), from where they infect the salivary glands (salivary gland invasion). Several gene expression systems, effectors and host factors can interfere with the different mosquito–parasite interactions throughout this cycle. Abbreviations: aapp, anopheline antiplatelet gene; act5C, actin 5C; Antryp1, late trypsin; Aper1, adult peritrophic matrix gene; Apy, apyrase; CecA, cecropin A; CEL-III, haemolytic C-type lectin; CP, zinc carboxypeptidase A1; CSPBP, CSP-binding protein; D7r, D7-related gene; ESP, epithelial serine protease; FREP1, fibrinogen-related protein 1; Lp, lipophorin; PLA2, bee venom phospholipase A2; SGS1, salivary gland surface protein 1; SM1, salivary gland midgut peptide 1; Vg, vitellogenin. Details on the expression systems listed are included in the text and the references cited relate to the first reported use in mosquitoes for the purpose of genetic modification.

(Ito *et al.*, 2002; Nolan *et al.*, 2011). The adult peritrophic matrix (*Aper1*) gene promoter has been used to direct constitutive midgut expression and is relevant to target initial stages of parasite development (Abraham *et al.*, 2005).

Approximately one day after blood ingestion, *Plasmodium* parasites cross the midgut as ookinetes, lodge under the basal lamina and develop into oocysts that face the haemocoel. Several thousand sporozoites develop in each oocyte and these are released into the haemolymph in the haemocoel of the insect. The vitellogenin (*Vg*) gene *cis*-acting sequences can be used in anophelines to induce late-digestion and sex-specific expression of desired gene products in the fat body for secretion into the haemolymph to target the sporozoites (Nirmala *et al.*, 2006; Chen *et al.*, 2007). Together, *CP* and *Vg* promoters are the most frequently used regulatory regions driving anti-parasite effector gene expression.

Additionally, effectors under the control of appropriate salivary gland-specific promoter regions can be used to drive expression before sporozoite invasion, including those from apyrase (*Apy*) and D7-related (*D7r*) genes (Lombardo *et al*., 2005), and, more strikingly, the anopheline antiplatelet gene (*aapp*) (Yoshida and Watanabe, 2006; Sumitani *et al*., 2013). Other control DNAs from the genes encoding a prophenoloxidase (PPO6, haemocyte-specific), actin5C (act5C, expressed constitutively in the midgut) and lipophorin (Lp, expressed constitutively in the fat body) may be convenient for alternative transgene expression strategies (Volohonsky *et al*., 2015). However, one hypothetical set of genes as yet to be identified are those that are tissue-specific and that respond uniquely to a parasite infection. In principle, if discovered, these would minimize even further the potential fitness costs associated with transgene expression – see examples below of the different degrees of fitness cost in genetically engineered mosquitoes.

Currently, genome modifications for engineering refractory *Anopheles* mosquitoes include an induced expression of exogenous or endogenous genes with known antipathogen effects, and/or gene editing of mosquito host factors required for parasite development. Many infection-blocking effectors are classified as exogenous lytic peptides, such as the scorpion venom protein, scorpine (Conde *et al*., 2000), bee venom phospholipase (*PLA*$_2$) (Moreira *et al*., 2002), the antimicrobial peptide mellitin (Carter *et al*., 2013), sea cucumber haemolytic C-type lectin (CEL-III) (Yoshida *et al*., 2007) and the synthetic peptides Shiva1 (Yoshida *et al*., 2001), Vida3 (Arrighi *et al*., 2002) and TP10 (Arrighi *et al*., 2008). All have been expressed by transgenes in mosquitoes and successfully suppress *Plasmodium* development (Meredith *et al*., 2011; Dong *et al*., 2020a,b). Another class of exogenous effector molecules is designed to bind the parasites or mosquito tissues, preventing invasion and development following infection. One of first molecules of this type for a malaria parasite-blocking strategy, the salivary gland midgut peptide 1 (SM1), was selected from a bacteriophage library (Ghosh *et al*., 2001; Ito *et al*., 2002)

and binds both the midgut receptor EBP (enolase-binding protein) on the luminal side of the midgut, and the Saglin receptors on the distal lobes of the salivary glands, blocking parasite interaction with these tissues (Ghosh *et al*., 2009, 2011; Vega-Rodriguez *et al*., 2014). Furthermore, work leveraged from transmission-blocking vaccines led to the identification and synthesis of potent modified monoclonal antibodies, single-chain fragments (scFv), that are directed specifically against *Plasmodium* parasite antigens (Yoshida *et al*., 1999). The inaugural work showing the feasibility of using expression systems to limit mosquito vector competence achieved virus-mediated transient expression of an anti-sporozoite scFv, with a resulting reduced salivary gland infection of as much as 99.9% when compared with controls (Capurro *et al*., 2000). Transgenes expressing 1C3, 4B7, or 2A10 scFvs, the first two of which inhibit ookinete invasion of the midgut and the third sporozoite invasion of salivary glands, resulted in fewer *Plasmodium falciparum* oocysts in transgenic *Anopheles stephensi* lines and significantly decreased sporozoite mean intensities of infection in salivary glands (Isaacs *et al*., 2011). Alternatively, using antibodies against mosquito-specific epitopes (Barreau *et al*., 1995; Brennan *et al*., 2000), as well as the transgenic expression of the mouse gene *Bax*, which causes salivary cell death in mosquitoes (Yamamoto *et al*., 2016), can also inhibit *Plasmodium* progression through mosquitoes. When considering the fitness of these transposon-based effector-expressing transgenic lines, different combinations of molecules and expression systems were shown to have contrasting impacts on mosquito survival and consequent transgene integration into populations. For example, midgut expression of the bee venom PLA$_2$ or expression of the peptide SM1 driven by the *Vg* promoter were shown to impose a significant fitness load to transgenic mosquitoes (Moreira *et al*., 2004; Li *et al*., 2008). However, PLA$_2$-expressing mosquitoes seem to have an advantage when fed *P. falciparum*-infected blood (Smith *et al*., 2013) and *CP*-driven expression of SM1 does not impact fitness of transgenic females (Moreira *et al*., 2004).

Besides, presumably due to parasite-target specificity, expression of a dual scFv transgene can completely inhibit *P. falciparum* development without significantly affecting fitness cost of the mosquitoes (Isaacs *et al.*, 2012). Single synthetic peptide (Vida3) expression in the female midgut did not affect fitness parameters of the transgenic population (McArthur *et al.*, 2014), whereas expression of multiple toxins and synthetic molecules with broader activity can exert undesired impacts on crucial physiological processes or on the gut microbiota (Dong *et al.*, 2020a,b).

The effectiveness of the endogenous mosquito response against parasites can be boosted by the transcriptional induction of immune effectors or the repression of negative regulators of immunity, as proved over the years through the use of transient reverse genetics (Frolet *et al.*, 2006; Garver *et al.*, 2009, 2013; Clayton *et al.*, 2013). The yellow fever mosquito, *Aedes aegypti*, was the first engineered genetically stable transgenic mosquito with an element of systemic immunity (Defensin A) activated through a blood-meal-triggered cascade (Kokoza *et al.*, 2000). Since then, successful demonstrations that endogenous immune effectors can be expressed in transgenic *Anopheles* mosquitoes include the overexpression of the NF-κB transcription factor Rel2 (Dong *et al.*, 2011), the anti-microbial peptide cecropin A (CecA) (Kim *et al.*, 2004), as well as the co-expression of these in multi-effector strategies (Isaacs *et al.*, 2012; Dong *et al.*, 2020a,b), all of which strongly reduce parasite numbers in salivary glands. However, overexpression of the anti-parasitic protein TEP1 in *An. gambiae* does not result in increased resistance to *Plasmodium* (Volohonsky *et al.*, 2017), demonstrating that simply augmenting the level of a given immune factor may not be sufficient to achieve greater resistance levels.

Another class of immune regulators that have significance in pathogen infection in mosquitoes is microRNAs (miRNAs). miRNAs have been shown in *An. gambiae* to function as both immune agonists and antagonists, regulating *Plasmodium* infection (Biryukova *et al.*, 2014; Dennison *et al.*, 2015). Transgenic depletion of specific miRNAs transcriptionally induced several immunity genes and increased

mosquito refractoriness to *Plasmodium* with minimal effect on fitness parameters (Dong *et al.*, 2020a,b). In addition, some metabolic interventions, for example genetic manipulation of the insulin pathway regulator, Akt, elicit mitochondrial dysfunction that enhances parasite killing in the midgut, but also shortens mosquito lifespan (Corby-Harris *et al.*, 2010). In contrast, overexpression of an inhibitor of the same pathway (PTEN) extends mosquito lifespan and increases resistance to *P. falciparum* development, by improving the integrity of the midgut barrier (Hauck *et al.*, 2013). These data demonstrate that while there is promise in immune-modulating intervention strategies, exploring different aspects of vector biology as well as a greater understanding of mosquito–parasite interactions can be useful to develop efficient and targeted genetic control strategies (Shaw and Catteruccia, 2019; Talyuli *et al.*, 2021).

The association between *Plasmodium* and *Anopheles* species has resulted in some mosquito genes being required for successful parasite infection and development in the vector host (Simões *et al.*, 2018). Key examples include the previously mentioned Saglin, which promotes salivary gland invasion (Ghosh *et al.*, 2009), as well as *Lp* and *Vg*, which reduce parasite-killing efficiency of TEP1 (Rono *et al.*, 2010). The geographical compatibility of *P. falciparum* strains and *Anopheles* species based on parasite–mosquito receptor ligand-like interactions provides an opportunity for increased specificity in the development of infection-blocking strategies (Molina-Cruz *et al.*, 2015, 2020). Although parasite–host interactions can be limited by genetic targeting of host factors, complete disruption of these can be challenging. Inactivation of the fibrinogen-related protein 1 (FREP1) gene via CRISPR/Cas9 gene editing, while suppressing infection with malaria parasites, also results in a wide array of fitness costs for the mosquito (Dong *et al.*, 2018). Yang *et al.* (2020), using a similar CRISPR-related approach, showed a failure to obtain a homozygous knockout mosquito following the complete deletion of the *mosGILT* gene. However, genetic mosaics with reduced mosGILT protein levels showed abnormal ovaries, but, importantly, also refractoriness

to parasite infection (Yang *et al*., 2020). Therefore, the exploration of mosquito *Plasmodium* agonists for the development of malaria control strategies based on parasite suppression has lagged behind other approaches, likely because of difficulties in generating benign, fitness-neutral targeting methods. This can be altered by future investigation of gene variants or the development of an efficient conditional knockout of host factors via bloodmeal-inducible expression of either the Cas9 protein or gRNAs in infection-relevant tissues (Xue *et al*., 2014). Given that *P. falciparum* is a highly polymorphic pathogen (Zhang *et al*., 2019), whose sequence diversity has been shown to limit effectiveness of single-target blocking strategies (Neafsey *et al*., 2015), it is important to consider exploring combinations of different effector molecules and also potentially targeting host factors required for propagation (Fig. 11.2).

11.4 Performance Objectives of Population Modification

The ultimate goal of all genetic control strategies is to reduce the number of infectious mosquitoes below a threshold level so that the probability of transmission falls to a point where the parasite population is too small to maintain the infection cycle. This has been defined as reducing the basic reproductive rate (R_0) of the disease below one ($R_0 < 1$) (Sinden, 2015). However, assessment of the necessary frequency and efficiency of a genetic modification capable of limiting a mosquito's infectious potential in the field is complicated. Typically, established concepts and metrics for measuring malaria transmission do not explicitly distinguish between light and heavy infections or the likelihood that a deemed infectious mosquito bite will actually result in a blood-stage infection in humans (Smith *et al*., 2012). This understudied area of malaria biology is of key importance to better appreciate the dynamics of infection in natural settings and predict the impact of genetic interventions (Graumans *et al*., 2020).

Early clinical and experimental evidence supported the idea that even a mosquito with a strongly lowered parasite burden can be infectious, since the small inoculum of only ten sporozoites is sufficient for infecting humans (Ungureanu *et al*., 1976) and low sporozoite numbers were implicated in an avian model of parasite transmission (Jasinskiene *et al*., 2007). Therefore, the fundamental thinking was that if only one ookinete successfully transposes the midgut barriers, develops into an oocyst and produces thousands of sporozoites, this should be enough to sustain infectiveness (Rosenberg *et al*., 1990). For many years this notion encouraged transgenic mosquito researchers to take the most stringent endpoint and set a goal for 'zero prevalence' of sporozoites in the salivary glands (Jasinskiene *et al*., 2007; Isaacs *et al*., 2012). However, classic reports in malaria epidemiology support the conclusion that the majority of infected mosquito bites may not result in a detectable infection (Davey and Gordon, 1933; Davidson and Draper, 1953; Pull and Grab, 1974). More recently, it has been shown with the rodent malaria model that mosquitoes with fewer *Plasmodium berghei* sporozoites ($\leq$ about 400 per salivary gland) are less infectious (Ito *et al*., 2002; Churcher *et al*., 2017). In a novel study, Aleshnick *et al*. (2020) proved experimentally that the relationship between mosquito salivary gland infection load and transmission probability is not linear and indeed must meet a threshold. The chance of infection increases particularly at a range between 10,000 and 20,000 sporozoites per salivary gland (Aleshnick *et al*., 2020). However, it is difficult to extrapolate model system transmission data directly to human malaria settings. One of the reasons is because the protocol used for the assessment of sporozoite infectivity in humans (in controlled malaria infections to evaluate vaccine efficacy) is carried out purposely using heavily infected mosquitoes, which is not reflective of parasite densities found in nature (Walk *et al*., 2018). In fact, most laboratory studies that describe efforts for malaria parasite suppression in mosquitoes also overestimate infection levels, likely underestimating the success rate of the resistance achieved. This is despite the fact that the data on the infection intensity in

naturally occurring infected mosquitoes indicate that the majority of them harbour a limited number of parasites (< 5 oocysts per gut and < 10,000 sporozoites per gland) (Pringle and Avery-Jones, 1966; Beier *et al.*, 1987; Billingsley *et al.*, 1994; Gouagna *et al.*, 2014). In addition, the effectiveness of transmission-blocking vaccine candidates in vertebrates is considered to be regulated tightly by mosquito parasite density, with the antibodies being more efficient at lower forces of infection in the mosquito (Bompard *et al.*, 2017; Churcher *et al.*, 2017). To evaluate accurately and correctly the potential of a given transmission-blocking strategy, measurements compatible with a natural infection system, with control parasite levels within the range of those found in wild-caught insects, are needed for genetically engineered mosquitoes. It is not yet sufficiently defined whether a 'no sporozoite' phenotype is indeed necessary to significantly impact malaria transmission, therefore interventions that decrease the mean intensity of infection might be as important as those that reduce parasite prevalence in the mosquito (Graumans *et al.*, 2020).

It appears intuitive that the expression of multiple anti-*Plasmodium* transgenes in different mosquito tissues would result in an additive effect and potentiate the level of refractoriness to parasite infection. It was observed in mice immunizations that the combination of two partially effective anti-malarial antibodies do achieve synergy in efficacy upon lower mosquito parasite loads (Sherrard-Smith *et al.*, 2018). This indicates both that a similar improved effect might be expected when combining effectors for mosquito refractoriness, and that interventions aimed to reduce infection intensity can be useful to aid in disease elimination strategies that combine vector modification and host-vaccination efforts. Nevertheless, it is important to consider that in genetically engineered insects, a single transgene that produces polycistronic mRNAs can result in reduced levels of each of the effectors compared with what could be achieved through single-effector constructs (Daniels *et al.*, 2014). Dong *et al.* (2020a,b) reported that combinations of endogenous and exogenous

effectors were able to induce highly potent suppression of parasite load and infection prevalence. However, this is not valid for all the effector combinations, and reproductive fitness and mosquito survival can be impaired significantly in some multi-effector transgenic lines (Dong *et al.*, 2020a,b). Spatio-temporal expression of multiple anti-parasitic effectors targeting different *Plasmodium* stages should still be a goal, because it limits the probability of emergence of parasite resistance. Furthermore, strategic planning for malaria control should include multiple parameters and be adjusted to the level of transmission-blocking efficacy required, given that even short-effect interventions could eliminate *Plasmodium* from vector and host populations in low transmission settings (Blagborough *et al.*, 2013).

Perhaps the most attractive feature of population modification gene drive systems is the possibility of creating a high-impact, low-cost and sustainable tool for controlling disease transmission (Carballar-Lejarazú and James, 2017). Whether gene drives are predicted to succeed in wild populations depends on two key parameters: the homing efficiency and fitness, meaning the relative fecundity or death rate the drive and its cargo confer on the modified organisms compared with the wild-type counterparts. Therefore, drives are favoured by selection if the inheritance bias of the drive exceeds its fitness penalty (Noble *et al.*, 2018). In fact, the first population modification by CRISPR-based gene drive in *Anopheles* achieved a proof-of-concept drive efficacy despite a substantial fitness cost of female mosquitoes homozygous for the drive (Gantz *et al.*, 2015). A carefully considered genome target may prevent an unintended disruption of important or essential genes and associated fitness loads. For this, the primary insertion site can be tested for its impact on fitness, as discussed previously. Furthermore, the effectors produced may exert physiological imbalance, or transgene expression might usurp resources needed for normal survival or reproductive functions (Terenius *et al.*, 2008). A number of life-table parameters must be determined under conditions that better mimic the natural environment of the

mosquitoes and the release strategy proposed (Facchinelli *et al.*, 2019; Pham *et al.*, 2019). Given that the higher rate of inheritance associated with effective gene drive systems renders them capable of increasing in frequency up to fixation in the population, the concept of a necessary complete fitness neutrality in modified mosquitoes can be reasonably challenged on an approach for implementation (James *et al.*, 2018).

Finally, it is important to discuss issues that are not yet fully understood or cannot be experimentally predicted in modification efforts. One puzzling example is the 'suppression escape phenotype' on modified lines that exhibit an overall strong refractoriness, represented by the few individual mosquitoes that present high levels of parasite infection (Isaacs *et al.*, 2012; Dong *et al.*, 2020a,b). Phenotypic variability and incomplete penetrance are frequently observed in transgenic animals (DeLoia and Solter, 1990; Pereira *et al.*, 1994; Kearns *et al.*, 2000; Seda *et al.*, 2019). In the case of effector-expressing mosquito populations, environmental and epigenetic factors could contribute to exceptional lowered effector expression or immune-suppressed phenotypes in some individuals. It is also possible that parasite variations (e.g., polymorphisms, developmental deviations) would make them spatio-temporally circumvent the expression of transgenic effectors. However, it is unclear whether this heterogeneity could lead to a modified dominant pattern of inheritance in the mosquito population or accelerate the emergence of parasite resistance. Certainly, parasite selection or evolution of resistance to transgenic blocking mechanisms are important subjects to address, because no efficient animal models exist yet for studying transmission of human malaria parasites. As mentioned above, population genetics mathematical models are needed to demonstrate the future dynamics and nature of the proposed systems and whether they exhibit robustness to imperfect homing, incomplete penetrance and transgene fitness costs, each of which is of practical significance given that real-world components inevitably have such imperfections. Furthermore, it is of paramount importance to address not only questions from the scientific community, but also concerns expressed by the public and the media about the potential ecological, ethical and social impact of gene drive modification systems, in order to consider regulatory approval prior to any field trials (Singh, 2019).

11.5 Conclusions

CRISPR/Cas9 systems have revolutionized the ability to produce genetics-based tools to add to the current incomplete toolkit for disease elimination and malaria eradication goals. Among the applications, coupling anti-pathogenic transgenes to gene drive systems has a strong potential to combat vector-borne diseases, due to their combined ability to spread into natural populations and block pathogen transmission. Population modification strategies should not be seen as a single solution, but as a component of a set of robust new methods that, integrated with current tools, should improve outcomes towards the elimination of malaria. The greatest opportunity for impact on eradication is a better understanding of vector–parasite interactions and transmission features, as well as genetic systems in mosquitoes, that may be used for the development and validation of novel disease-control tools.

Malaria control presents variable challenges across its transmission spectrum and strategic planning for elimination should consider a number of factors, with particular emphasis on the transmission-blocking effectiveness required and the transmission intensity in the targeted area. Regarding the development framework, a target product profile helps researchers identify their specific goals and realistic go/no-go criteria for efficacy of an investigational product before moving further along the testing pathway (James *et al.*, 2018). Evidence of laboratory efficacy, as well as fitness, safety, release strategies and minimally acceptable performance parameters, provide the basis for evaluating novel technologies for field use (Carballar-Lejarazú *et al.*, 2020; Long *et al.*, 2021). The design and field implementation of a population modification product is

likely to be both complex and multifaceted, although current data suggest that we may be closer than we previously thought to the utilization of an effective and safe anti-malarial technology that might reverse the current global disease trend.

Acknowledgements

References

Abraham, E.G., Donnelly-Doman, M., Fujioka, H., Ghosh, A., Moreira, L., and Jacobs-Lorena, M. (2005) Driving midgut-specific expression and secretion of a foreign protein in transgenic mosquitoes with AgAper1 regulatory elements. *Insect Molecular Biology* 14, 271–279. doi:10.1111/j.1365-2583.2004.00557.x

Adolfi, A., Gantz, V.M., Jasinskiene, N., Lee, H., Hwang, K., Terradas, G. *et al.* (2020) Efficient population modification gene-drive rescue system in the malaria mosquito *Anopheles stephensi*. *Nature Communications* 11, 5553. doi:10.1038/s41467-020-19426-0

Aleshnick, M., Ganusov, V.V., Nasir, G., Yenokyan, G. and Sinnis, P. (2020) Experimental determination of the force of malaria infection reveals a non-linear relationship to mosquito sporozoite loads. *PLoS Pathogens* 16, 1–23. doi:10.1371/journal.ppat.1008181

Arrighi, R.B.G., Nakamura, C., Miyake, J., Hurd, H. and Burgess, J.G. (2002) Design and activity of antimicrobial peptides against sporogonic-stage parasites causing murine malarias. *Antimicrobial Agents and Chemotherapy* 46, 2104–2110. doi: 10.1128/AAC.46.7.2104-2110.2002

Arrighi, R.B.G., Ebikeme, C., Jiang, Y., Ranford-Cartwright, L., Barrett, M.P. *et al.* (2008) Cell-penetrating peptide TP10 shows broad-spectrum activity against both *Plasmodium falciparum* and *Trypanosoma brucei brucei*. *Antimicrobial Agents and Chemotherapy* 52, 3414–3417. doi:10.1128/AAC.01450-07.

Barreau, C., Touray, M., Pimenta, P.F., Miller, L.H. and Vernick, K.D. (1995) *Plasmodium gallinaceum*: sporozoite invasion of *Aedes aegypti* salivary glands is inhibited by anti-gland antibodies and by lectins. *Experimental Parasitology* 81, 332–343. doi: 10.1006/expr.1995.1124

Beaghton, A., Hammond, A., Nolan, T., Crisanti, A., Godfray, H.C.J. and Burt, A. (2017) Requirements for driving antipathogen effector genes into populations of disease vectors by homing. *Genetics* 205, 1587–1596. doi: 10.1534/genetics.116.197632

Beier, J.C., Whitmire, R.E., Mugambi, M., Perkins, P.V, Wirtz, R.A., and Hockmeyer, W.T. (1987) Field evaluation of an enzyme-linked immunosorbent assay (ELISA) for *Plasmodium falciparum* sporozoite detection in anopheline mosquitoes from Kenya. *American Journal of Tropical Medicine and Hygiene* 36, 459–468. doi: 10.4269/ajtmh.1987.36.459

Bhatt, S., Weiss, D.J., Cameron, E., Bisanzio, D., Mappin, B. *et al.* (2015) The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature* 526, 207–211. doi: 10.1038/nature15535

Billingsley, P.F., Sinden, R.E., Medley, G.F. and Charlwood, D. (1994) Relationship between prevalence and intensity of *Plasmodium falciparum* infection in natural populations of *Anopheles* mosquitoes. *American Journal of Tropical Medicine and Hygiene* 51, 260–270. doi: 10.4269/ajtmh.1994.51.260

Biryukova, I., Ye, T. and Levashina, E. (2014) Transcriptome-wide analysis of microRNA expression in the malaria mosquito *Anopheles gambiae*. *BMC Genomics* 15, 557. doi: 10.1186/1471-2164-15-557

Blagborough, A.M., Churcher, T.S., Upton, L.M., Ghani, A.C., Gething, P.W., and Sinden, R.E. (2013). Transmission-blocking interventions eliminate malaria from laboratory populations. *Nature Communications* 4, 1812. doi: 10.1038/ncomms2840

Bompard, A., Da, D.F, Yerbanga, R.S., Biswas, S., Kapulu, M. *et al.* (2017) Evaluation of two lead malaria transmission blocking vaccine candidate antibodies in natural parasite–vector combinations. *Scientific Reports* 7, 1–9. doi: 10.1038/s41598-017-06130-1

Brennan, J.D.G., Kent, M., Dhar, R., Fujioka, H. and Kumar, N. (2000) *Anopheles gambiae* salivary gland proteins as putative targets for blocking transmission of malaria parasites. *Proceedings of the National Academy of Sciences USA* 97, 13859–13864. doi: 10.1073/pnas.250472597

Burt, A. (2003) Site-specific selfish genes as tools for the control and genetic engineering of natural populations. *Proceedings of the Royal Society B: Biological Sciences* 270, 921–928. doi: 10.1098/rspb.2002.2319

Capurro, M.L., Coleman, J., Beerntsen, B.T., Myles, K.M., Olson, K.E. *et al.* (2000) Virus-expressed, recombinant single-chain antibody blocks sporozoite infection of salivary glands in *Plasmodium*

gallinaceum-infected *Aedes aegypti*. *American Journal of Tropical Medicine and Hygiene* 62, 427–33. doi: 10.4269/ajtmh.2000.62.427

Caragata, E.P., Dong, S., Dong, Y., Simões, M.L., Tikhe, C.V. and Dimopoulos, G. (2020) Prospects and pitfalls: next-generation tools to control mosquito-transmitted disease. *Annual Review of Microbiology* 74, 455–475. doi: 10.1146/annurev-micro-011320-025557

Carballar-Lejarazú, R. and James, A.A. (2017) Population modification of Anopheline species to control malaria transmission. *Pathogens and Global Health* 111, 424–435. doi: 10.1080/20477724.2018.1427192

Carballar-Lejarazú, R., Ogaugwu, C., Tushar, T., Kelsey, A., Pham, T.B. *et al.* (2020). Next-generation gene drive for population modification of the malaria vector mosquito, *Anopheles gambiae*. *Proceedings of the National Academy of Sciences* 117, 22805–22814. doi: 10.1073/pnas.2010214117

Carter, V., Underhill, A., Baber, I., Sylla, L., Baby, M. *et al.* (2013) killer bee molecules: antimicrobial peptides as effector molecules to target sporogonic stages of *Plasmodium*. *PLoS Pathogens* 9, 1–13. doi: 10.1371/journal.ppat.1003790

Chen, X.G., Marinotti, O., Whitman, L., Jasinskiene, N. and James, A.A. (2007) The *Anopheles gambiae* vitellogenin gene (VGT2) promoter directs persistent accumulation of a reporter gene product in transgenic *Anopheles stephensi* following multiple bloodmeals. *American Journal of Tropical Medicine and Hygiene* 76, 1118–1124. doi: 10.4269/ajtmh.2007.76.1118

Chen, X.-G., Mathur, G. and James, A.A. (2008) Gene expression studies in mosquitoes. *Advances in Genetics* 54, 19–50. doi: 10.1016/S0065-2660(08)00802-X

Churcher, T.S., Sinden, R.E., Edwards, N.J., Poulton, I.D., Rampling, T.W. *et al.* (2017) Probability of transmission of malaria from mosquito to human is regulated by mosquito parasite density in naïve and vaccinated hosts. *PLoS Pathogens* 13, 1–18. doi: 10.1371/journal.ppat.1006108

Clayton, A.M., Cirimotich, C.M., Dong, Y. and Dimopoulos, G. (2013) Caudal is a negative regulator of the *Anopheles* IMD pathway that controls resistance to *Plasmodium falciparum* infection. *Developmental and Comparative Immunology* 39, 323–332. doi: 10.1016/j.dci.2012.10.009

Conde, R., Zamudio, F.Z., Rodrıguez, M.H. and Possani, L.D. (2000). Scorpine, an anti-malaria and anti-bacterial agent purified from scorpion venom. *FEBS Letters* 471, 165–168. doi: 10.1016/S0014-5793(00)01384-3

Corby-Harris, V., Drexler, A., de Jong, L.W., Antonova, Y., Pakpour, N. *et al.* (2010) Activation of akt signaling reduces the prevalence and intensity of malaria parasite infection and lifespan in *Anopheles stephensi* mosquitoes. *PLoS Pathogens* 6, 1–10. doi: 10.1371/journal.ppat.1001003

Curtis, C.F. (1968) Possible use of translocations to fix desirable genes in insect pest populations. *Nature* 218, 368–9. doi: 10.1038/218368a0

Curtis, C.F. (1992) Selfish genes in mosquitoes. *Nature* 357, 450–450. doi: 10.1038/357450b0

Daniels, R.W., Rossano, A.J., Macleod, G.T. and Ganetzky, B. (2014) Expression of multiple transgenes from a single construct using viral 2a peptides in Drosophila. *PLoS ONE* 9, e100637. doi: 10.1371/journal.pone.0100637

Davey, T.H. and Gordon, R.M. (1933) The estimation of the density of infective anophelines as a method of calculating the relative risk of inoculation with malaria from different species or in different localities. *Annals of Tropical Medicine & Parasitology* 27, 27–52. doi: 10.1080/00034983.1933.11684737

Davidson, G., and Draper, C.C. (1953) Field studies of some of the basic factors concerned in the transmission of malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 47, 522–535. doi: 10.1016/S0035-9203(53)80005-2

DeLoia, J.A. and Solter, D. (1990) A transgene insertional mutation at an imprinted locus in the mouse genome. *Development (Suppl.)* 1990, 73–79.

Dennison, N.J., BenMarzouk-Hidalgo, O.J. and Dimopoulos, G. (2015) MicroRNA-regulation of *Anopheles gambiae* immunity to *Plasmodium falciparum* infection and midgut microbiota. *Developmental and Comparative Immunology* 49, 170–178. doi: 10.1016/j.dci.2014.10.016

Dong, Y., Das, S., Cirimotich, C., Souza-Neto, J.A., McLean, K.J., and Dimopoulos, G. (2011) Engineered *Anopheles* immunity to *Plasmodium* infection. *PLoS Pathogens* 7, e1002458. doi: 10.1371/journal.ppat.1002458

Dong, Y., Simões, M.L., Marois, E. and Dimopoulos, G. (2018) CRISPR/Cas9 -mediated gene knockout of *Anopheles gambiae* FREP1 suppresses malaria parasite infection. *PLoS Pathogens* 14, 1–16. doi: 10.1371/journal.ppat.1006898.

Dong, S., Fu, X., Dong, Y., Simões, M.L., Zhu, J., and Dimopoulos, G. (2020a) Broad spectrum immunomodulatory effects of *Anopheles gambiae* microRNAs and their use for transgenic suppression of *Plasmodium*. *PLOS Pathogens* 16, e1008453. doi: 10.1371/journal.ppat.1008453

Dong, Y., Simões, M.L. and Dimopoulos, G. (2020b) Versatile transgenic multistage effector-gene combinations for *Plasmodium falciparum* suppression in *Anopheles*. *Science Advances* 6, eaay5898. doi: 10.1126/sciadv.aay5898

Facchinelli, L., North, A.R., Collins, C.M., Menichelli, M., Persampieri, T. *et al.* (2019) Large-cage assessment of a transgenic sex-ratio distortion strain on populations of an African malaria vector. *Parasites and Vectors* 12, 1–14. doi: 10.1186/s13071-019-3289-y

Frolet, C., Thoma, M., Blandin, S., Hoffmann, J.A. and Levashina, E.A. (2006) Boosting NF-κB-dependent basal immunity of *Anopheles gambiae* aborts development of *Plasmodium berghei*. *Immunity* 25, 677–685. doi: 10.1016/j.immuni.2006.08.019

Gantz, V.M. and Bier, E. (2015) The mutagenic chain reaction: A method for converting heterozygous to homozygous mutations. *Science* 348, 442–444. doi: 10.1126/science.aaa5945

Gantz, V.M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V.M. *et al.* (2015) Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. *Proceedings of the National Academy of Sciences USA* 112, e6736–e6743. doi: 10.1073/pnas.1521077112

Garver, L.S., Dong, Y. and Dimopoulos, G. (2009) Caspar controls resistance to *Plasmodium falciparum* in diverse anopheline species. *PLoS Pathogens* 5, e1000335. doi: 10.1371/journal.ppat.1000335

Garver, L.S., de Almeida Oliveira, G. and Barillas-Mury, C. (2013) The JNK pathway is a key mediator of *Anopheles gambiae* antiplasmodial immunity. *PLoS Pathogens* 9, e1003622. doi: 10.1371/journal.ppat.1003622.

Ghosh, A.K., Ribolla, P.E.M. and Jacobs-Lorena, M. (2001) Targeting *Plasmodium* ligands on mosquito salivary glands and midgut with a phage display peptide library. *Proceedings of the National Academy of Sciences USA* 98, 13278–13281. doi: 10.1073/pnas.241491198.

Ghosh, A K., Devenport, M., Jethwaney, D., Kalume, D E., Pandey, A. *et al.* (2009) Malaria parasite invasion of the mosquito salivary gland requires interaction between the *Plasmodium* TRAP and the *Anopheles* saglin proteins. *PLoS Pathogens* 5, e1000265. doi: 10.1371/journal.ppat.1000265.

Ghosh, A.K., Coppens, I., Gårdsvoll, H., Ploug, M. and Jacobs-Lorena, M. (2011) *Plasmodium* ookinetes coopt mammalian plasminogen to invade the mosquito midgut. *Proceedings of the National Academy of Sciences USA* 108, 17153–17158. doi: 10.1073/pnas.1103657108.

Gouagna, L.C., Yao, F., Yameogo, B., Dabiré, R.K. and Ouédraogo, J.B. (2014) Comparison of field-based xenodiagnosis and direct membrane feeding assays for evaluating host infectiousness to malaria vector *Anopheles gambiae*. *Acta Tropica* 130, 131–139. doi: 10.1016/j.actatropica.2013.10.022

Graumans, W., Jacobs, E., Bousema, T. and Sinnis, P. (2020) When Is a *Plasmodium*-infected mosquito an infectious mosquito? *Trends in Parasitology* 36, 705–716. doi: 10.1016/j.pt.2020.05.011

Häcker, I., and Schetelig, M.F. (2018) Molecular tools to create new strains for mosquito sexing and vector control. *Parasites & Vectors* 11, 645. doi: 10.1186/s13071-018-3209-6

Hammond, A.M. and Galizi, R. (2017) Gene drives to fight malaria: current state and future directions. *Pathogens and Global Health* 111, 412–423. doi: 10.1080/20477724.2018.1438880

Hauck, E.S., Antonova-Koch, Y., Drexler, A., Pietri, J., Pakpour, N. *et al.* (2013) Overexpression of phosphatase and tensin homolog improves fitness and decreases *Plasmodium falciparum* development in *Anopheles stephensi*. *Microbes and Infection* 15, 775–787. doi: 10.1016/j.micinf.2013.05.006

Isaacs, A.T., Li, F., Jasinskiene, N., Chen, X., Nirmala, X. *et al.* (2011) Engineered resistance to *Plasmodium falciparum* development in transgenic *Anopheles stephensi*. *PLoS Pathogens* 7, e1002017. doi: 10.1371/journal.ppat.1002017

Isaacs, A.T., Jasinskiene, N., Tretiakov, M., Thiery, I., Zettor, A. *et al.* (2012) Transgenic *Anopheles stephensi* coexpressing single-chain antibodies resist *Plasmodium falciparum* development. *Proceedings of the National Academy of Sciences USA* 109, E1922–E1930. doi: 10.1073/pnas.1207738109

Ito, J., Ghosh, A., Moreira, L.A., Wimmer, E.A. and Jacobs-Lorena, M. (2002) Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature* 417, 452–455. doi: 10.1038/417452a

James, S., Collins, F.H., Welkhoff, P.A., Emerson, C., Godfray, H.C.J. *et al.* (2018) Pathway to deployment of gene drive mosquitoes as a potential biocontrol tool for elimination of malaria in Sub-Saharan Africa: recommendations of a Scientific Working Group. *American Journal of Tropical Medicine and Hygiene* 98, 1–49. doi: 10.4269/ajtmh.18-0083

Jasinskiene, N., Coleman, J., Ashikyan, A., Salampessy, M., Marinotti, O., and James, A.A. (2007) Genetic control of malaria parasite transmission: threshold levels for infection in an avian model system. *American Journal of Tropical Medicine and Hygiene* 76, 1072–1078. doi: 10.4269/ajtmh.2007.76.1072

Kearns, M., Preis, J., McDonald, M., Morris, C. and Whitelaw, E. (2000) Complex patterns of inheritance of an imprinted murine transgene suggest incomplete germline erasure. *Nucleic Acids Research* 28, 3301–3309. doi: 10.1093/nar/28.17.3301

Kim, W., Koo, H., Richman, A.M., Seeley, D., Vizioli, J. *et al.* (2004). Ectopic expression of a cecropin transgene in the human malaria vector mosquito *Anopheles gambiae* (Diptera: Culicidae): effects on susceptibility to *Plasmodium*. *Journal of Medical Entomology* 41, 447–455. doi: 10.1603/0022-2585-41.3.447

Kokoza, V., Ahmed, A., Cho, W.-L., Jasinskiene, N., James, A.A., and Raikhel, A. (2000) Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. *Proceedings of the National Academy of Sciences USA* 97, 9144–9149. doi: 10.1073/pnas.160258197

Li, C., Marrelli, M.T., Yan, G. and Jacobs-Lorena, M. (2008) Fitness of transgenic *Anopheles stephensi* mosquitoes expressing the SM1 peptide under the control of a vitellogenin promoter. *Journal of Heredity* 99, 275–282. doi: 10.1093/jhered/esn004

Lombardo, F., Nolan, T., Lycett, G., Lanfrancotti, A., Stich, N. *et al.* (2005) An *Anopheles gambiae* salivary gland promoter analysis in *Drosophila melanogaster* and *Anopheles stephensi*. *Insect Molecular Biology* 14, 207–216. doi: 10.1111/j.1365-2583.2004.00549.x

Long, K.C., Alphey, L., Annas, G.J., Bloss, C.S., Campbell, K J. *et al.* (2021) Core commitments for field trials of gene drive organisms. *Science* 370, 1417–1419. doi: 10.1126/science.abd1908

McArthur, C.C., Meredith, J.M. and Eggleston, P. (2014) Transgenic *Anopheles gambiae* expressing an antimalarial peptide suffer no significant fitness cost. *PLoS ONE* 9, e88625. doi: 10.1371/journal.pone.0088625

Meredith, J.M., Basu, S., Nimmo, D.D., Larget-Thiery, I., Warr, E.L. *et al.* (2011) Site-specific integration and expression of an anti-malarial gene in transgenic *Anopheles gambiae* significantly reduces *Plasmodium* infections. *PLoS ONE* 6, 1–9. doi: 10.1371/journal.pone.0014587

Molina-Cruz, A., Canepa, G.E., Kamath, N., Pavlovic, N.V., Mu, J. *et al.* (2015) *Plasmodium* evasion of mosquito immunity and global malaria transmission: the lock-and-key theory. *Proceedings of the National Academy of Sciences USA* 112, 15178–15183. doi: 10.1073/pnas.1520426112

Molina-Cruz, A., Canepa, G.E., Alves, E., Silva, T.L., Williams, A.E. *et al.* (2020) *Plasmodium falciparum* evades immunity of anopheline mosquitoes by interacting with a Pfs47 midgut receptor. *Proceedings of the National Academy of Sciences USA* 117, 2597–2605. doi: 10.1073/pnas.1917042117

Moreira, L.A., Ito, J., Ghosh, A., Devenport, M., Zieler, H. *et al.* (2002) Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes. *Journal of Biological Chemistry* 277, 40839–40843. doi: 10.1074/jbc.M206647200

Moreira, L.A., Wang, J., Collins, F.H. and Jacobs-Lorena, M. (2004) Fitness of anopheline mosquitoes expressing transgenes that inhibit *Plasmodium* development. *Genetics* 166, 1337–1341. doi: 10.1534/genetics.166.3.1337

Neafsey, D.E., Juraska, M., Bedford, T., Benkeser, D., Valim, C. *et al.* (2015) Genetic diversity and protective efficacy of the RTS,S/AS01 malaria vaccine. *New England Journal of Medicine* 373, 2025–2037. doi: 10.1056/NEJMoa1505819

Nirmala, X., Marinotti, O., Sandoval, J.M., Phin, S., Gakhar, S. *et al.* (2006) Functional characterization of the promoter of the vitellogenin gene, AsVg1, of the malaria vector, *Anopheles stephensi*. *Insect Biochemistry and Molecular Biology* 36, 694–700. doi: 10.1016/j.ibmb.2006.05.011

Noble, C., Olejarz, J., Esvelt, K.M., Church, G.M. and Nowak, M.A. (2017) Evolutionary dynamics of CRISPR gene drive. *Science Advances* 3, 3–10. doi: 10.1126/sciadv.1601964

Noble, C., Adlam, B., Church, G.M., Esvelt, K.M. and Nowak, M.A. (2018) Current CRISPR gene drive systems are likely to be highly invasive in wild populations. *Elife* 7, 1–30. doi: 10.7554/eLife.33423

Nolan, T., Petris, E., Müller, H.-M., Cronin, A., Catteruccia, F. and Crisanti, A. (2011) Analysis of two novel midgut-specific promoters driving transgene expression in *Anopheles stephensi* mosquitoes. *PLoS ONE* 6, e16471. doi: 10.1371/journal.pone.0016471

Pereira, R., Halford, K., Sokolov, B.P., Khillan, J.S. and Prockop, D.J. (1994). Phenotypic variability and incomplete penetrance of spontaneous fractures in an inbred strain of transgenic mice expressing a mutated collagen gene (COL1A1). *Journal of Clinical Investigation* 93, 1765–1769. doi: 10.1172/JCI117161

Pham, T.B., Phong, C.H., Bennett, J.B., Hwang, K., Jasinskiene, N. *et al.* (2019) Experimental population modification of the malaria vector mosquito, *Anopheles stephensi*. *PLoS Genetics* 15, e1008440. doi: 10.1371/journal.pgen.1008440

Pringle, G. and Avery-Jones, S. (1966) An assessment of the sporozoite inoculation rate as a measure of malaria transmission in the Ubembe area of North-east Tanzania. *Journal of Tropical Medicine and*

Hygiene 69, 132–139. Available at: https://pubmed.ncbi.nlm.nih.gov/5940666/ (accessed 20 April 2021).

Pull, J.H. and Grab, B. (1974) A simple epidemiological model for evaluating the malaria inoculation rate and the risk of infection in infants. *Bulletin of the World Health Organization* 51, 507–516. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4549501 (accessed April 2021)

Ran, F.A.F.A., Hsu, P.D.P., Wright, J., Agarwala, V., Scott, D.A. and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols* 8, 2281–2308. doi: 10.1038/nprot.2013.143

Robert, M.A., Legros, M., Facchinelli, L., Valerio, L., Ramsey, J.M. *et al.* (2012) Mathematical models as aids for design and development of experiments: the case of transgenic mosquitoes. *Journal of Medical Entomology* 49, 1177–1188. doi: 10.1603/ME11205

Rono, M.K., Whitten, M.M.A., Oulad-Abdelghani, M., Levashina, E.A. and Marois, E. (2010) The major yolk protein vitellogenin interferes with the anti-plasmodium response in the malaria mosquito anopheles gambiae. *PLoS Biology* 8, e1000434. doi: 10.1371/journal.pbio.1000434

Rosenberg, R., Wirtz, R.A., Schneider, I. and Burge, R. (1990) An estimation of the number of malaria sporozoites ejected by a feeding mosquito. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 84, 209–212. doi: 10.1016/0035-9203(90)90258-G

Schmidt, H., Collier, T.C., Hanemaaijer, M.J., Houston, P.D., Lee, Y. and Lanzaro, G.C. (2020) Abundance of conserved CRISPR-Cas9 target sites within the highly polymorphic genomes of *Anopheles* and *Aedes* mosquitoes. *Nature Communications* 11, 1–6. doi: 10.1038/s41467-020-15204-0

Seda, M., Peskett, E., Demetriou, C., Bryant, D., Moore, G.E. *et al.* (2019) Analysis of transgenic zebrafish expressing the Lenz-Majewski syndrome gene PTDSS1 in skeletal cell lineages. *F1000Research* 8, 273. doi: 10.12688/f1000research.17314.1

Shaw, W.R. and Catteruccia, F. (2019) Vector biology meets disease control: using basic research to fight vector-borne diseases. *Nature Microbiology* 4, 20–34. doi: 10.1038/s41564-018-0214-7

Sherrard-Smith, E., Sala, K.A., Betancourt, M., Upton, L.M., Angrisano, F. *et al.* (2018) Synergy in anti-malarial pre-erythrocytic and transmission-blocking antibodies is achieved by reducing parasite density. *Elife* 7, 1–15. doi: 10.7554/eLife.35213

Simões, M.L., Caragata, E.P. and Dimopoulos, G. (2018) Diverse host and restriction factors regulate mosquito–pathogen interactions. *Trends in Parasitology* 34, 603–616. doi: 10.1016/j.pt.2018.04.011

Simoni, A., Siniscalchi, C., Chan, Y.-S., Huen, D.S., Russell, S. *et al.* (2014) Development of synthetic selfish elements based on modular nucleases in *Drosophila melanogaster*. *Nucleic Acids Research* 42, 7461–7472. doi: 10.1093/nar/gku387

Sinden, R.E. (2015) The cell biology of malaria infection of mosquito: advances and opportunities. *Cellular Microbiology* 17, 451–466. doi: 10.1111/cmi.12413

Singh, J.A. (2019) Informed consent and community engagement in open field research: lessons for gene drive science. *BMC Medical Ethics* 20, 54. doi: 10.1186/s12910-019-0389-3

Smith, D.L., Battle, K.E., Hay, S.I., Barker, C.M., Scott, T.W. and McKenzie, F.E. (2012) Ross, Macdonald, and a theory for the dynamics and control of mosquito-transmitted pathogens. *PLoS Pathogens* 8, e1002588. doi: 10.1371/journal.ppat.1002588

Smith, R.C., Kizito, C., Rasgon, J.L. and Jacobs-Lorena, M. (2013) Transgenic mosquitoes expressing a phospholipase A2 gene have a fitness advantage when fed *Plasmodium falciparum*-infected blood. *PLoS ONE* 8, 1–11. doi: 10.1371/journal.pone.0076097

Sreenivasamurthy, S.K., Dey, G., Ramu, M., Kumar, M., Gupta, M.K. *et al.* (2013) A compendium of molecules involved in vector–pathogen interactions pertaining to malaria. *Malaria Journal* 12, 1–7. doi: 10.1186/1475-2875-12-216

Sumitani, M., Kasashima, K., Yamamoto, D.S., Yagi, K., Yuda, M. *et al.* (2013) Reduction of malaria transmission by transgenic mosquitoes expressing an antisporozoite antibody in their salivary glands. *Insect Molecular Biology* 22, 41–51. doi: 10.1111/j.1365-2583.2012.01168.x

Talyuli, O.A.C., Bottino-Rojas, V., Polycarpo, C.R., Oliveira, P.L. and Paiva-Silva, G.O. (2021) Non-immune traits triggered by blood intake impact vectorial competence. *Frontiers in Physiology* 12, 1–21. doi: 10.3389/fphys.2021.638033

Terenius, O., Marinotti, O., Sieglaff, D. and James, A.A. (2008) Molecular genetic manipulation of vector mosquitoes. *Cell Host and Microbe* 4, 417–423. doi: 10.1016/j.chom.2008.09.002

Unckless, R.L., Clark, A.G. and Messer, P.W. (2017) Evolution of resistance against CRISPR/Cas9 gene drive. *Genetics* 205, 827–841. doi: 10.1534/genetics.116.197285

Ungureanu, E., Killick-Kendrick, R., Garnham, P.C.C., Branzei, P., Romanescu, C. and Shute, P.G. (1976) Prepatent periods of a tropical strain of *Plasmodium vivax* after inoculations of tenfold dilutions of

sporozoites. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 70, 482–483. doi: 10.1016/0035-9203(76)90133-4

Vega-Rodriguez, J., Ghosh, A.K., Kanzok, S.M., Dinglasan, R.R., Wang, S. *et al.* (2014) Multiple pathways for *Plasmodium* ookinete invasion of the mosquito midgut. *Proceedings of the National Academy of Sciences USA* 111, E492–E500. doi: 10.1073/pnas.1315517111

Volohonsky, G., Terenzi, O., Soichot, J., Naujoks, D.A., Nolan, T. *et al.* (2015) Tools for *Anopheles gambiae* transgenesis. *G3 Genes, Genomes, Genetics* 5, 1151–1163. doi: 10.1534/g3.115.016808

Volohonsky, G., Hopp, A.K., Saenger, M., Soichot, J., Scholze, H. *et al.* (2017) Transgenic expression of the anti-parasitic factor TEP1 in the malaria mosquito *Anopheles gambiae*. *PLoS Pathogens* 13, 1–26. doi: 10.1371/journal.ppat.1006113

Walk, J., Van Gemert, G.J., Graumans, W., Sauerwein, R.W. and Bijker, E.M. (2018) Mosquito infectivity and parasitemia after controlled human malaria infection. *American Journal of Tropical Medicine and Hygiene* 98, 1705–1708. doi: 10.4269/ajtmh.17-0952

World Health Organization (2020) World malaria report 2020: 20 years of global progress and challenges. *Global Malarial Programme*. Available at: https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2020.

Xue, Z., Wu, M., Wen, K., Ren, M., Long, L. *et al.* (2014) CRISPR/Cas9 mediates efficient conditional mutagenesis in *Drosophila*. *G3: Genes, Genomes, Genetics* 4, 2167–2173. doi: 10.1534/g3.114.014159

Yamamoto, D.S., Sumitani, M., Kasashima, K., Sezutsu, H. and Matsuoka, H. (2016) Inhibition of malaria infection in transgenic anopheline mosquitoes lacking salivary gland cells. *PLoS Pathogens* 12, 1–21. doi: 10.1371/journal.ppat.1005872

Yang, J., Schleicher, T.R., Dong, Y., Park, H.B., Lan, J. *et al.* (2020). Disruption of mosGILT in *Anopheles gambiae* impairs ovarian development and *Plasmodium* infection. *Journal of Experimental Medicine* 217, 1–13. doi: 10.1084/jem.20190682

Yoshida, S. and Watanabe, H. (2006) Robust salivary gland-specific transgene expression in *Anopheles stephensi* mosquito. *Insect Molecular Biology* 15, 403–410. doi: 10.1111/j.1365-2583.2006.00645.x

Yoshida, S., Ioka, D., Matsuoka, H., Endo, H. and Ishii, A. (2001) Bacteria expressing single-chain immuno-toxin inhibit malaria parasite development in mosquitoes. *Molecular and Biochemical Parasitology* 113, 89–96. doi: 10.1016/S0166-6851(00)00387-X

Yoshida, S., Matsuoka, H., Luo, E., Iwai, K., Arai, M. *et al.* (1999) A single-chain antibody fragment specific for the *Plasmodium berghei* ookinete protein Pbs21 confers transmission blockade in the mosquito midgut. *Molecular and Biochemical Parasitology* 104, 195–204.

Yoshida, S., Shimada, Y., Kondoh, D., Kouzuma, Y., Ghosh, A.K. *et al.* (2007) Hemolytic C-type lectin CEL-III from sea cucumber expressed in transgenic mosquitoes impairs malaria parasite development. *PLoS Pathogens* 3, 1962–1970. doi: 10.1371/journal.ppat.0030192

Zhang, X., Alexander, N., Leonardi, I., Mason, C., Kirkman, L.A. and Deitsch, K.W. (2019) Rapid antigen diversification through mitotic recombination in the human malaria parasite *Plasmodium falciparum*. *PLoS Biology* 17, e3000271. doi: 10.1371/journal.pbio.3000271

12 Modelling Threshold-Dependent Gene Drives: a Case Study Using Engineered Underdominance

Matthew P. Edgington and Luke Alphey*
The Pirbright Institute, Woking, Surrey, UK

12.1 Introduction to Threshold-Dependent Gene Drives

Gene drives have been proposed as valuable tools in the fight against a range of globally important issues, including vectors of disease, invasive species and agricultural pests. These approaches are classified primarily based on their persistence and/or invasiveness. Here we consider persistent (i.e. self-sustaining) and low-invasiveness (i.e. threshold-dependent) approaches using engineered underdominance as a case study.

Gene drive is a phenomenon whereby a particular gene (or suite of genes) can bias inheritance in its (their) own favour, thus allowing it to increase in frequency over successive generations, even when deleterious to carrier individuals (Sinkins and Gould, 2006; Alphey, 2014, 2020; Champer *et al.*, 2016; NASEM, 2016; Leftwich *et al.*, 2018;) (see Raban and Akbari, Chapter 8; Champer, Chapter 9, this volume). This may occur by a range of natural or synthetic mechanisms, most of which act in one of two ways: (i) conversion of progeny individuals into other genotypes; or (ii) reducing the fitness (or killing) of progeny individuals of certain genotypes.

The precise configuration of gene drive components can lead to systems with a wide range of different behavioural characteristics, and these are often used to classify the various gene drive systems. Perhaps the most common classifications are based on their intended purpose (usually population modification or suppression), invasiveness (ability to spread into non-target populations) and persistence (whether they remain in the population or diminish over time).

Owing mainly to coverage in popular media outlets, the term gene drive is often associated with only widely known systems such as some CRISPR-based homing approaches. These are expected to have relatively straightforward behaviour in that they are highly invasive (spreading from extremely small releases and so likely also to spread to all populations linked by any degree of gene flow), highly persistent (at least in absence of resistance) and able to be used flexibly for either population suppression or modification (see Bottino-Rojas and James, Chapter 11, this volume). As discussed previously (James, 2005; NASEM, 2016; Harvey-Samuel *et al.*, 2019; Long *et al.*, 2020; Lanzaro *et al.*, 2021), the first gene

---

*Corresponding author, email: luke.alphey@pirbright.ac.uk

drive trials are likely to be conducted in remote areas such as highly isolated islands to limit the probability of spill-over into non-target populations. It is debatable whether geographical containment of this type is adequate for non-localized (sometimes also referred to as 'global') gene drive approaches, as it would be difficult to guarantee perfect confinement within the trial area. Another potential drawback is that any gene drive releases would presumably need to gain regulatory approval from all affected countries, which for global systems could be argued to be all countries in which the target species (and any capable of forming fertile hybrids) are present (see Beech *et al.*, Chapter 25, this volume). For the first gene drive trial releases, such widespread regulatory approval required for a global system would seem challenging to obtain, at least for widely distributed species. Localized gene drives may need approval only in the target territory and may be particularly suitable where homogenous modification of every population of the species is not desired.

Though not as widely discussed in the media as non-localized drives, several designs for localized drives have been proposed and subject to considerable analysis. Here we focus on two-locus engineered underdominance (UD) – an example of threshold-dependent gene drive that should be persistent, reversible and spatially restricted. This would appear to answer much of the concern around non-localized gene drive approaches, since the system is unlikely to spread significantly beyond any initial trial site and can be reversed easily in the event of unintended consequences – features likely to prove desirable to regulatory bodies and other stakeholders in the context of initial proof-of-concept gene drive field trials.

In this chapter, we outline a range of modelling approaches that have been used to demonstrate the key characteristics of this approach, including threshold introduction frequencies, reversibility, spatial limitation and robustness to mutation/resistance. We then go on to discuss alternative configurations based on the use of sex-specific components and their effect on introduction thresholds. We conclude with a discussion

on the cycle of information between mathematical models and experimental data along with a range of areas for future modelling that will be important in providing information on the anticipated effects of these systems when released into target populations.

12.2 Two-Locus Engineered Underdominance

Underdominance, also known as negative heterosis, is a natural phenomenon and the opposite of the better-known overdominance (positive heterosis or hybrid vigour). Thus, underdominance occurs where hybrids are of lower fitness than either of two different true-breeding parental strains; for practical purposes, one of these parental strains is wild-type, the other is the underdominance-based (UD) gene drive strain. In a single locus scenario, modelling of such selection against hybrids has been shown to allow for the eventual fixation of one allele, with the other being eliminated (Wilson and Turelli, 1986; Altrock *et al.*, 2010, 2011). More recently, transgenes displaying these properties have been developed and tested (Reeves *et al.*, 2014; Maselko *et al.*, 2020; Buchman *et al.*, 2021). The UD concept can also be expanded beyond a single locus. UD gene drives can potentially be developed using transgenic constructs containing toxin and antidote components. The particular configuration considered here is two-locus UD as originally proposed by Davis *et al.* (2001). This approach requires two distinct transgenic constructs to be inserted at independently segregating (unlinked) genomic loci, each of which comprises a lethal effector (toxin) and a suppressor (antidote) for the toxin of the other transgenic construct (Fig. 12.1). One (or optionally both) of these transgenic constructs will also contain a genetic cargo aimed at producing a desirable phenotype within the target population, for example a reduced ability to transmit a given pathogen (e.g. Franz *et al.*, 2006, 2014; Buchman *et al.*, 2019) (see Franz, Chapter 22, this volume). This combination of transgenic components gives an underdominance-like
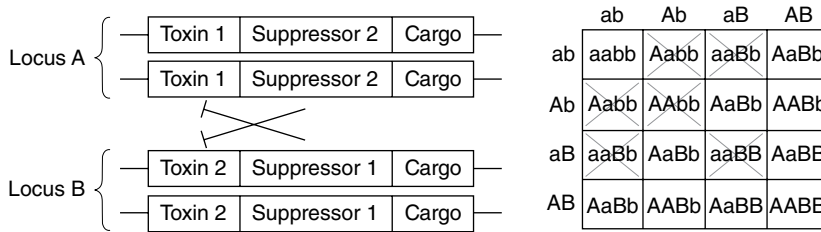
Fig. 12.1. A schematic diagram illustrating the workings of a two-locus engineered underdominance gene drive system. (Left) This gene drive design requires the introduction of two distinct transgenic constructs at independently segregating genomic loci. Each transgenic construct comprises a toxin, an element suppressing the toxin of the other transgenic construct and a desirable genetic cargo (which may be included within one or both constructs). **(Right)** A Punnett square demonstrating the creation of an underdominance-like effect. Here haplotypes outside the square represent gametes from a viable parent (one maternal and one paternal). The resulting offspring genotypes are listed inside the square and it is here that lethality manifests in individuals carrying just one of the transgenic constructs (grey crosses), creating a selective pressure for individuals to carry either both transgenic constructs or neither.

effect by creating a negative selection (via a lethal effect) on individuals carrying just one of the transgenic constructs (since they contain a toxin but not the antidote from the other transgenic construct). This results in a positive selection pressure for individuals to carry either both or neither of the transgenic constructs (Fig. 12.1). The precise strength of this selection pressure is dependent on several factors, including the degree of lethality conferred on affected genotypes and the fitness cost caused by the presence of the gene drive constructs, for example imperfect suppression of the lethals by the suppressors, or insertional effects of the transgene on nearby genes.

12.3 Mathematical Modelling Approaches

Building gene drives in the laboratory is an inherently time-consuming and expensive activity. Mathematical modelling, on the other hand, can be conducted relatively quickly and, by comparison, inexpensively and allows systematic exploration of parameter space far more readily than empirical methods. It is therefore extremely beneficial to model any proposed gene drive approach in advance of (or at least concurrently with) the laboratory development of transgenic

components, and this has indeed been widespread practice. Models can be used to determine essential performance targets that must be met for engineered gene drives to achieve their intended function, particularly within laboratory-based experiments or field-based trial releases. The structure and complexity of such gene drive models can vary dramatically, with each having their own respective benefits and limitations. It is here that experienced mathematical modellers are key in determining the most appropriate model to use in any given scenario while understanding and being able to communicate to non-modellers how a given model structure may influence modelling outcomes. It seems intuitive that mathematical modelling has the potential to save vast amounts of experimental time, effort and money where gene drive designs and engineered components are not fit for purpose. Perhaps less obvious is the requirement for models, and model-based conclusions, to be used (or at least scrutinized) by experienced modellers who understand the impact of model assumptions, complexity and limitations.

A variety of mathematical modelling approaches have been used to provide insight into the predicted performance of UD gene drives; however, much of this work has focused on deterministic population genetics

models. These can provide insight into the basic function and utility of UD systems. Such models typically consider an idealized scenario consisting of an infinitely large population (avoiding stochastic effects and the need for integer numbers of individuals) that is isolated (closed from any migration) and panmictic (randomly mating). For simplicity, it is also commonly assumed that females mate only once and produce a 1:1 (female to male) sex ratio in their offspring. Attention is typically restricted to the case whereby no resistance mechanisms can emerge and where each component of the introduced transgenes is immutable and perfectly linked (i.e., toxin, antidote and cargo genes are unable to separate from one another). Finally, it is commonly assumed that generations of offspring in modelled populations are synchronous (i.e., non-overlapping), which may not always be realistic but can apply to laboratory caged populations or wild populations that are synchronized by climatic factors (e.g., wet and dry seasons). This allows for the use of simple recurrence relations (i.e., difference equations) to model the population genetics resulting from the release of such a gene drive. This typical set of simplifying assumptions is also adopted in the example below.

Much of the modelling of UD gene drives uses genotype-based population genetics models. In the case of a two-locus approach such as UD, this results in a total of nine possible genotypes – homozygous, heterozygous or wild-type for the transgene at each of two loci – and therefore a set of nine difference equations. However, since UD is based entirely on Mendelian inheritance and lethality to certain offspring genotypes, the offspring in each generation are directly related to the proportions of each allele present in the parental generation rather than the precise parental genotypes. This allows the consideration of a simpler model that requires the tracking of only the four haplotype frequencies (ab, Ab, aB and AB, where a/b represent wild-type alleles and A/B their transgenic counterparts). This results in a set of four difference equations that may be solved recursively in a manner similar to that originally presented by Davis et al. (2001). Magori and Gould (2006) considered a similar model structure but additionally allowed for multiple insertions of each transgene, though this is not included here. This model is of the form:

$$ab_{t+1} = \frac{\left[\left(ab_t^{\,2}\right) + \left(\frac{1}{2}\varepsilon^2 ab_t\, AB_t\right) + \left(\frac{1}{2}\varepsilon^2 aB_t\, Ab_t\right)\right]}{\bar{\Omega}} = \frac{f_1}{\bar{\Omega}},$$

(Equation 1)

$$aB_{t+1} = \frac{\left[\left(\frac{1}{2}\varepsilon^2 ab_t\, AB_t\right) + \left(\frac{1}{2}\varepsilon^2 aB_t\, Ab_t\right) + \left(\varepsilon^3 aB_t\, AB_t\right)\right]}{\bar{\Omega}} = \frac{f_2}{\bar{\Omega}},$$

(Equation 2)

$$Ab_{t+1} = \frac{\left[\left(\frac{1}{2}\varepsilon^2 ab_t\, AB_t\right) + \left(\frac{1}{2}\varepsilon^2 aB_t\, Ab_t\right) + \left(\varepsilon^3 Ab_t\, AB_t\right)\right]}{\bar{\Omega}} = \frac{f_3}{\bar{\Omega}},$$

(Equation 3)

$$AB_{t+1} = \frac{\left[\left(\frac{1}{2}\varepsilon^2 ab_t\, AB_t\right) + \left(\frac{1}{2}\varepsilon^2 aB_t\, Ab_t\right) + \left(\varepsilon^3 aB_t\, AB_t\right) + \left(\varepsilon^3 Ab_t\, AB_t\right) + \left(\varepsilon^4 AB_t^{\,2}\right)\right]}{\bar{\Omega}} = \frac{f_4}{\bar{\Omega}},$$

(Equation 4)

where

$$\bar{\Omega} = f_1 + f_2 + f_3 + f_4,$$

(5)

is the sum of numerators in (Equation 1)–(Equation 4), ε denotes the fitness (relative to wild-type) of an individual carrying a transgenic construct and t denotes the generation from which the next allele frequency is computed. For simplicity, here we assume that each transgenic construct (A and B) confers the same degree of fitness cost on carrier individuals and that these are applied multiplicatively where individuals carry more than one transgenic construct (up to a maximum of four, where the relative fitness would be given by ε^4). Note that the parameter ε can take any value in the range from zero (completely non-viable) to one (equally as fit as wild-type) and that the consideration of multiplicative relative fitness ensures that the overall value for any genotype also remains in the range from zero to one. Like much of the modelling literature, here we assume that toxins are fully penetrant (i.e., no viable offspring result) and similar for antidotes (i.e., a single antidote copy provides full rescue).

The model presented here provides one of the simplest possible models of UD gene drive and is useful for determining various base-level characteristics of the system. As with all models, this is based on a range of simplifying assumptions (described above), each of which is likely to have its own implications. There is a wide range of other modelling approaches that can be (and have been) used to capture the anticipated effects of relaxing one or more of these model assumptions. Several of these are briefly discussed in the following sections, focusing primarily on results rather than extensive modelling detail. Modelling of UD systems has spanned a range of model structures, including difference equations, ordinary differential equations (ODEs), delay differential equations (DDEs), partial differential equations (PDEs) and stochastic models, each of which provides insights into different aspects of anticipated UD behaviour.

12.4 Introduction Thresholds

Underdominance acting at a single locus has been shown to produce bistable dynamics:

either homozygotic state can be stable, depending on the initial frequencies and relative fitness of each type, as shown for underdominant alleles (Wilson and Turelli, 1986; Altrock *et al.*, 2010, 2011) and chromosome translocations (Curtis, 1968). UD gene drives seek to capture a similar effect synthetically, via the introduction of toxin and antidote elements. While the threshold dependence of UD has been demonstrated using numerical simulation under a range of release sizes, to our knowledge a full equilibrium analysis has yet to be conducted. This can be achieved either analytically or computationally and here we focus on the latter, using the numerical continuation software package XPPAUT (Ermentrout, 2002), producing results shown in Fig. 12.2.

These results show two distinct regimes of behaviour separated by a particular relative fitness parameter $\varepsilon^* \approx 0.725$ (as these are applied multiplicatively, this gives UD double homozygotes a relative fitness of just ~ 0.276). In the region $\varepsilon < \varepsilon^*$ there are two possible equilibrium states, with either the wild-type (stable) or gene drive (unstable) alleles at fixation. This can be interpreted as a scenario in which the gene drive is unable to establish itself, no matter how many gene drive-carrying individuals are introduced. The unstable equilibrium whereby gene drive alleles are at fixation is not biologically feasible, since it would imply there were no wild-type individuals present at the time of release – rendering the release of a gene drive unnecessary. The more interesting region ($\varepsilon > \varepsilon^*$) displays four possible equilibrium states. The first is the unstable (and not biologically feasible) equilibrium state with gene drive alleles at fixation. The three remaining equilibria together constitute a bistable system (i.e. two stable equilibria separated by an unstable equilibrium). Focusing on the gene drive allele (Fig. 12.2c) below the unstable equilibrium, the elimination of the gene drive is the only stable equilibrium – representing negative selection against the gene drive when introduced at a sub-threshold frequency. Above the unstable equilibrium, the gene drive moves towards a stable equilibrium with high gene drive frequency – representing positive
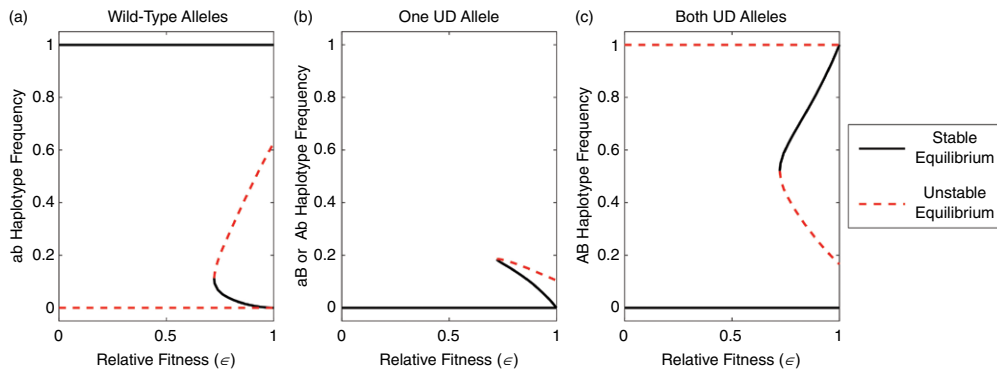
Fig. 12.2. A bifurcation diagram showing the possible equilibria of a two-locus engineered underdominance gene drive and their associated stability properties. Here stable equilibria are shown by solid black lines while unstable equilibria are shown by red dashed lines, with panel (a) showing ab (i.e. fully wild-type), (b) Ab or aB (i.e. a single UD allele) and (c) AB (i.e. both UD alleles) haplotype frequencies. These diagrams show the bistable nature of this gene drive approach. When introduced above a certain threshold, the gene drive system increases in frequency, towards the stable equilibrium with non-zero AB haplotype frequency. When introduced below this threshold, the system decreases in frequency, heading towards the stable equilibrium with a zero AB haplotype frequency. These diagrams also demonstrate the existence of a maximum tolerable fitness cost for two-locus engineered underdominance gene drive systems of around 28% per construct. Bifurcation analysis was conducted using XPPAUT continuation software (Ermentrout, 2002) and results were plotted using MATLAB (R2020b, The MathWorks Inc., Natick, Massachusetts).

selection when introducing the gene drive above the threshold frequency. Theoretically it is possible that the system would attain the unstable equilibrium state and remain there; however practically this is exceedingly unlikely, due to the many and varied stochastic effects present in the real world.

A feature evident in the results of Fig. 12.2 is that the equilibrium state for a UD system does not necessarily comprise only gene drive homozygotes. Where there are no fitness costs associated with the gene drive, the system can reach fixation (i.e. 100% gene drive homozygotes). However, where fitness costs are non-zero, wild-type alleles are expected to be present at a frequency that increases with the fitness costs of the system (Fig. 12.2b).

While useful in displaying the bistable (i.e. threshold-dependent) nature of a UD gene drive, Fig. 12.2 is not necessarily of direct use when planning a gene drive release. This is due to the combination of AB and Ab/aB haplotypes present at the unstable equilibrium (i.e. the introduction threshold). In practice it would be more convenient

to know a single gene drive frequency above which the system must be introduced for it to increase in frequency within the target population. Fortunately, this can be calculated by summing to obtain the overall gene drive allele frequency for each point on the unstable equilibrium line. This results in a single threshold gene drive allele frequency (as shown in Fig. 12.3) that must be exceeded through any combination of heterozygote or homozygote individuals. Note that these results align with the pattern observed in several previous studies (e.g. Magori and Gould, 2006; Edgington and Alphey, 2017, 2018; Dhole *et al.*, 2018, 2020; Leftwich *et al.*, 2018), though precise thresholds may differ due to assumptions on the application of fitness costs to individuals carrying multiple transgenic constructs and the choice of presentation method.

12.5   Relaxing Model Assumptions

As discussed above, gene drive models are based on a range of simplifying assumptions,
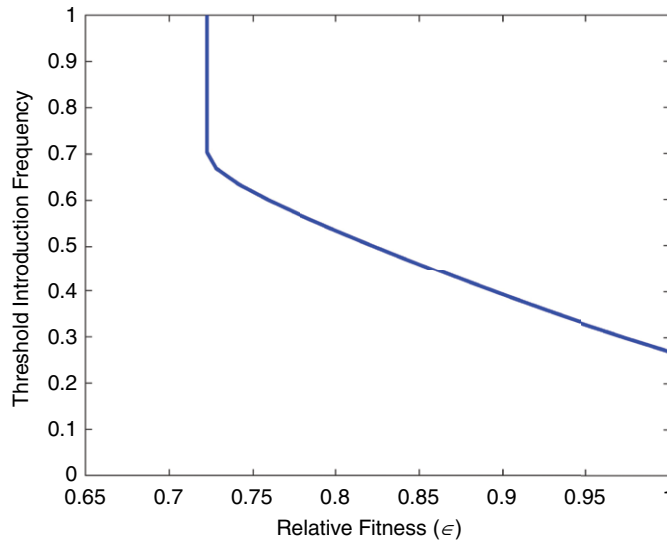
Fig. 12.3. Threshold introduction frequencies required for an engineered underdominance system to spread in a target population over a range of relative fitness parameters. Note that this figure was generated by summing gene drive allele frequencies from results in Fig. 12.2, but mirror those previously shown in Edgington and Alphey (2017, 2018), using alternative mathematical models – although some slight differences are observed due to assumptions on how fitness costs should be applied to individuals carrying multiple transgenic constructs.

each of which has its own implications for the outcomes and applicability of models to different scenarios. The model outlined in section 12.3 represents one of the simplest useful representations of a UD gene drive system and, as shown in section 12.4, allows key characteristics of this gene drive design to be elucidated. Of course, this model structure can be altered to allow for the relaxation of any of the model assumptions outlined above, thereby allowing their implications to be explored. In the following sections we discuss studies exploring the relaxation of three such model assumptions: (1) the presence of resistance formation and mutation of transgenic constructs; (2) the reversal of UD gene drives; and (3) the incorporation of spatial effects.

12.5.1 Resistance formation and mutation

A common set of simplifying assumptions for gene drive modelling studies is that elements within a single transgenic construct are perfectly linked (i.e., unable to separate), do not undergo mutation (i.e., lose function of transgenic components) and that no other resistance mechanisms emerge. Edgington and Alphey (2019) relaxed this assumption by modelling a scenario whereby transgenic constructs accumulate loss-of-function mutations at a constant rate. To our knowledge, rates of mutation in insects likely to be targeted by gene drives are not well studied and could vary considerably, depending on the molecular biology of the gene drive system. Thus, mutation rates (per gene) are assumed to fall within the range 10^{-4}–10^{-8} that should span rates relevant to a range of target insect species. This parameter range is based on measured mutation rates in *Drosophila melanogaster* (estimated as being of the order 10^{-9} per nucleotide per generation) (Haag-Liautard et al., 2007; Keightley et al., 2014); the size of gene drive constructs in previous studies (∼1–10 kb) (Windbichler et al., 2011; Reeves et al., 2014; Champer et al., 2017) and an
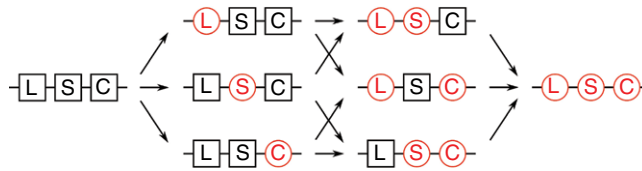
Fig. 12.4. Transgenic constructs are assumed to mutate at a rate of *m* per gene. This is assumed to be low enough that multiple mutations per generation may be neglected. For example, the initial transgenic construct (say, A) mutates at a rate of $3m$, producing mutations in the lethal (giving A_L), suppressor (A_S) and the cargo (A_C) gene each at a rate of m. Then, transgenic constructs possessing one mutated gene (e.g. A_L) mutate at a rate of $2m$ (giving A_{LS} and A_{LC} each at rate m). Transgenic constructs with two mutated genes (e.g. A_{LS}) then mutate at rate m, producing constructs with all three genes mutated (i.e. A_{LSC}). Here, non-mutated genes are represented by black squares whereas genes with loss-of-function mutations are shown in red circles. (Figure originally published in Edgington and Alphey, 2019.)

assumption that ∼1–10% of nucleotides in transgenic constructs are essential for gene function. Note that these mutation rates are assumed to be low enough that multiple mutations (i.e., in more than one gene) within a single generation may be neglected, leading to the pattern of mutation shown in Fig. 12.4.

The original study describing this scheme of mutation (Edgington and Alphey, 2019) considered a genotype-based formulation, resulting in a set of 2025 genotypes, of which 819 were non-viable. This could be reduced to a haplotype-based formulation with 81 haplotypes (81 difference equations), making the model simpler and faster to formulate, code and simulate.

This study found that UD displays an increase in frequency that is almost completely unaffected by such mutation where mutated transgenic constructs conferred a greater fitness cost than their non-mutated counterparts – such cases will not be discussed further. Loss-of-function mutations are therefore only of concern where mutated transgenic constructs are of higher fitness than non-mutated versions. Here, the introduced UD system would initially increase in frequency if introduced above the required threshold. Over time each type of mutated transgenic construct will begin to accumulate, with the rate of accumulation and maximum frequencies varying depending on which loss-of-function mutations are present. Results in Edgington and Alphey (2019) show that, for a range of mutation rates and fitness costs, it is transgenic constructs with a single loss-of-function mutation in

either the lethal or cargo gene that achieve the greatest maximal frequencies (Fig. 12.5). Constructs with loss-of-function in two genes achieve lower frequencies and are dominated by those where the antidote (suppressor) gene is unaffected. Combined, the mutated transgenic constructs reach high overall frequencies, with a concurrent decrease in the frequency of non-mutated constructs. Since UD approaches typically achieve an equilibrium in which wild-type alleles remain present (see Fig. 12.2), these begin to replace the mutated transgenes due to their relative fitness advantage, eventually returning the population to a fully wild-type state (Fig. 12.5). It is yet to be studied in depth whether the stable co-existence of mutated and non-mutated transgenic constructs is possible, but it was not observed under any parameter set or initial condition considered in Edgington and Alphey (2019).

For different fitness costs and mutation rates, the observed dynamics remained broadly similar to those in Fig. 12.5, though the timescales and maximal frequencies of each mutation vary. Higher mutation rates reduce the period over which the UD system persists at high frequency – essentially the period in which the gene drive would maintain efficacy. Even though UD systems can be eliminated by mutations, they are predicted to remain at high frequency for hundreds of generations – likely long enough for the system to have produced its desired effect. As an example, *Aedes aegypti* mosquitoes (vectors of dengue, Zika, yellow fever and chikungunya viruses) undergo approximately
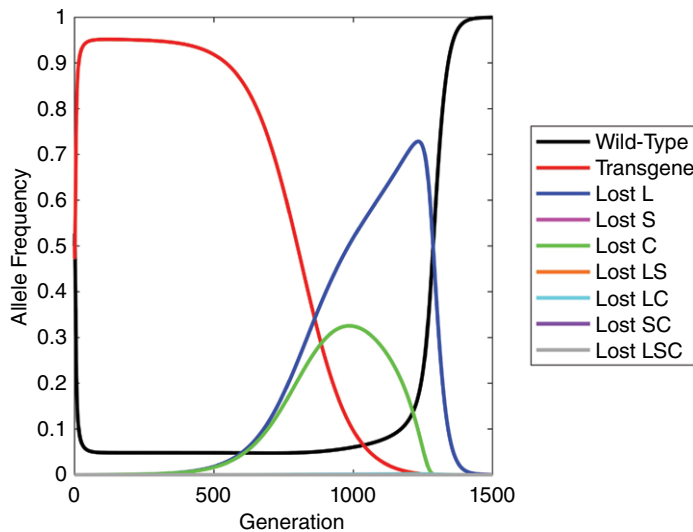
Fig. 12.5. An example numerical simulation showing the effects of mutation within transgenic constructs of an engineered underdominance gene drive system. Results here are presented for a 1:1 (introduced to wild) introduction of non-mutated double homozygote (AABB) individuals into a wild-type (aabb) population. Note that several alleles only reach very low maximum frequencies and thus they appear to overlie one another along the horizontal axis. Results are shown for an engineered underdominance system with 5% fitness cost per non-mutated construct, a 4% fitness cost per mutated construct and a mutation rate of $m = 10^{-6}$.

ten generations per year, meaning that the introduced UD system should persist at high frequency for at least ten years, even in the face of accumulating loss-of-function transgene mutations.

12.5.2 UD reversal

Ideally, a gene drive, or any other intervention, should be simple to reverse in the event of any unintended and undesirable consequences. For threshold-dependent drives such as underdominance (UD), the simplest mechanism for this is the release of wild-type individuals. Releasing a sufficiently large number of wild-type individuals can push the UD system below the threshold frequency, thus it undergoes negative selection and is driven out of the population (consider, for example, Fig. 12.2). To our knowledge, only one alternative reversal mechanism has been proposed for reversing UD, namely releasing individuals carrying free suppressors

(i.e., individuals carrying just the antidote from one or both of the original transgenic constructs) (Edgington and Alphey, 2019). This was proposed since modelling of mutation in UD systems showed significantly greater accumulation of mutated constructs that retained function of the antidote gene, which undergo positive selection where a UD system is at high frequency. The same study also showed that if free-suppressor elements conferred a non-zero fitness cost, then the positive selection would be lost as the UD system became rare in the population, thus allowing wild-type alleles to outcompete the free suppressor element, returning to a fully wild-type state.

With there being two viable mechanisms for UD reversal, it is instructive to compare the two. Each has benefits, but which would be more useful in real-world scenarios? If a released UD system were to require reversal, then it would be reasonable to assume that introducing large numbers of wild-type individuals would be undesirable, as it could potentially increase the population

above pre-control levels, albeit transiently. Intensive suppression might reduce numbers in the entire population such that the necessary wild-type releases remain below pre-control levels, but that would have its own costs and issues. In some cases, issues around release of wild-type individuals could be alleviated somewhat by releasing individuals of a single sex (as discussed in Leftwich *et al.*, 2018). For example, in many insect species (and certainly many of those likely to be gene drive targets) females provide both the reproductive and epidemiological potential of the population; thus, releasing wild-type males should generally be quite benign.

The release of free-suppressor individuals provides an alternative reversal mechanism and addresses some of the issues associated with wild-type release(s) but also has some drawbacks of its own. For example, free-suppressor individuals are theoretically able to function from an extremely small release. In practice, it would be desirable to perform release(s) large enough to avoid stochastic loss at low frequencies. Despite this, it should still be feasible to release far fewer individuals than required for wild-type reversal and these may be of a single sex. However, there are also some potential drawbacks that need to be weighed against these benefits. Firstly, free-suppressor releases have been shown to function much more slowly than wild-type reversal. For instance, Edgington and Alphey (2019) show that a 2:1 (reversal to wild) release of wild-type individuals can (approximately) eliminate the UD gene drive in about 20 generations whereas an equal release of free suppressor individuals took about 130 generations to reach the same point, with about a further 150 generations required for the free-suppressor individuals to be (approximately) eliminated. Another potential issue is whether the appropriate regulatory body would approve the release of further transgenic insects if the original system required reversal due to unintended negative effects.

12.5.3 Spatial effects

A key feature of UD gene drives is their threshold-dependent nature, since this is often stated to be capable of preventing the system from establishing in non-target neighbouring populations. It may even prevent the system reaching an appreciable frequency, due to negative selection when present at sub-threshold frequencies. The modelling of such spatial factors is therefore important in assessing how well confined UD systems will remain and under what conditions this confinement could potentially fail. These questions are expected to prove important when seeking to attain regulatory approval for UD releases into the field; with highly robust confinement, regulatory approval far beyond the release site(s), for example regional or multi-national approval, may not be required, in contrast to current thought regarding more invasive approaches.

Spatial effects can conceivably be studied in a variety of ways, including *n*-deme population genetics (difference equations), *n*-deme population dynamics (ordinary or delay differential equations), reaction–diffusion (partial differential equations) or individual-based models. Each of these has been used in the study of gene drive approaches, although not all in the context of UD, and possesses its own positive and negative features. Here we discuss a range of these approaches in the context of UD gene drives, focusing primarily on findings rather than technical details. While we cover a broad range of studies here, this is by no means intended as a comprehensive review.

Perhaps the most commonly used technique for assessing spatial properties of gene drives are *n*-deme population genetics models. Briefly, these consider two or more demes (semi-isolated (sub)populations), each of which has its own set of difference equations of the form outlined in section 12.4. For simplicity, the literature primarily considers a scenario with just two demes. These models capture the migration of individuals between demes via a simple exchange of a proportion of individuals in each generation. It is commonly assumed that the two populations are of equal size, such that the number of each migrant type is simply based on the haplotype frequencies in each population – a reasonable assumption where large (modelled as infinite) populations

are considered. This approach has been used to consider spatial aspects for a wide range of gene drive classes, including UD (Marshall and Hay, 2012; Harvey-Samuel *et al.*, 2019; Edgington *et al.*, 2020b). One such model estimated that a UD system with a homozygote fitness cost of 5% (applied additively) and a bidirectional migration rate of 1% per generation would reach near-fixation in the target population (e.g. results in Fig. 12.2) but reaches a frequency of just 0.032 in a non-target neighbouring population (Marshall and Hay, 2012). The same work then went on to estimate that the same UD system would require a bidirectional migration rate of 4.3% per generation to become established in both populations, thus supporting the notion that UD is robustly confineable.

The approximation of equal population sizes has also been relaxed in a number of studies focusing both on UD (Dhole *et al.*, 2018) and on other gene drive classes (Dhole *et al.*, 2019, 2020). These studies include an approximate scaling of migration rates to account for the differences in respective population sizes that result from gene drive fitness cost and lethal effects. While this captures an additional level of realism absent in the non-scaled migration models, the results obtained do not suggest that this will have a large impact on the ability of UD systems to remain confined. In fact, we would suggest that this is likely to improve the confinement, since transgene fitness costs and lethal effects will reduce the target population size, meaning fewer migrants into the non-target population. However, this will likely increase the influence of wild-type migrants from the non-target population, potentially creating a necessity for slightly larger UD release(s) to ensure the system remains above the introduction threshold.

Several studies have also considered extensions to these population genetics models by taking account of various ecological factors affecting the life cycle and size of the target and non-target insect populations when subject to the release of a UD gene drive (e.g., Edgington and Alphey, 2018; Khamis *et al.*, 2018, 2020). These each consider their own model structures to capture density-dependent effects during the immature stages of the insect life cycle, each with its respective advantages and disadvantages. One density-dependence function used in such models is that of Maynard Smith and Slatkin (1973) and is of the form:

$$f(N) = \left(1 + \left(aN\right)^b\right)^{-1},$$

where N denotes the size of the insect population, a is a density parameter ($1/a$ relates to the number of breeding sites) and b defines the strength of density-dependent competition. This function is known to be flexible in that it can capture a range of density-dependence scenarios (Bellows, 1981) and has been used in the study of UD (Edgington and Alphey, 2018) and other gene drive classes (Alphey and Bonsall, 2014). This work outlines a variety of possibilities not extensively discussed in the results of population genetics models. In particular it identifies three possible outcomes of a UD release: (1) no introgression in either population; (2) establishment in both populations; and (3) introgression into the target population with extremely limited spread into the non-target population, with the latter usually considered the most desirable outcome for UD (Edgington and Alphey, 2018). Sánchez *et al.* (2020a) considered similar effects for UD^{MEL} and reciprocal chromosome translocations using a computational framework called MGDriveE (Sánchez *et al.*, 2020b; Wu *et al.*, 2021).

The above approaches consider spatial effects via an exchange of individuals between two (sub)populations, which are assumed to be well mixed. A possible extension to this work is to consider spatial effects explicitly, using a model defined over a continuous spatial domain (e.g., Champer *et al.*, 2020d). This work takes a fully computational approach, using an individual-based model implemented in the open-source software package SLiM (Haller and Messer, 2016, 2019). Here, two circular regions (subpopulations) are linked by a narrow migration corridor, with movement assumed to result from the birth of new offspring (Champer *et al.*, 2020d). This showed that UD is robust against re-invasion by wild-type but may

display a greater degree of invasiveness into neighbouring populations than predicted with the spatially implicit model structures discussed above. However, the narrow migration corridor essentially forces migrating individuals to encounter those moving in the opposite direction, creating an approximately linear boundary between the two populations – a scenario shown to facilitate easier gene drive invasion (Champer *et al.*, 2020d).

An alternative scenario would allow migration to occur over a wider space, meaning migrants encounter those moving in the opposite direction far less frequently, thus eliminating the linear boundary within the migration corridor. Here migrants would first encounter individuals when arriving at the boundary of the opposite population, meaning they would encounter either a very high or very low local gene drive frequency, likely producing results closer to those from spatially implicit model structures. Such variation highlights the importance of understanding a wide range of species, location and ecological traits when predicting the outcome of a real-world UD release.

While the above approaches assess the likelihood of UD invading non-target populations, spatial effects are also important in determining the ability of UD to spread in a given target population. This has also been addressed using a variety of different modelling approaches.

One possibility is to consider a lattice-based model in which the target region is discretized into a collection of cells, each containing a well-mixed pool of individuals (Huang *et al.*, 2011). Individuals move between cells according to a dispersal kernel, defining the probability of an individual moving between any two cells in the lattice on any given day. This model structure was used to compare the relative efficacy of two UD release methods: (i) release into one large area; and (ii) release into many smaller, equally distributed areas. Interestingly, this work showed that either release method could be more effective, depending on the degree of mobility exhibited by individuals and the fitness costs associated with the UD system.

The individual-based model of Champer *et al.* (2020d) has also been used to explore the ability of UD to spread within a single population. This considered two spatial scenarios based on the shape of the UD release area, namely a scenario with either a straight-line (linear) scenario or a circle dividing regions of high and low/zero gene drive frequency. Interestingly, the UD system was able to spread or persist more readily in the linear scenario than the circular one. This was proposed to be a result of the local gene drive frequency being lower for the circular case, since the wild-type partially wraps around the high UD region. However, we would expect this effect to diminish rapidly as the circular region increases in size (reducing the curvature of the boundary).

Finally, another potential approach for considering spatial effects in either single or linked populations is the use of reaction–diffusion equations (i.e., partial differential equation models). These have been explored in the context of highly invasive CRISPR-based gene drives (Beaghton *et al.*, 2016; Tanaka *et al.*, 2017), but to our knowledge have yet to be widely applied to threshold-dependent systems. Such models should enable a wide range of spatial scenarios to be considered, while allowing various sources of heterogeneity to be considered across the spatial domain. These models can also allow an explicit representation of insect migration to be incorporated into population dynamics model structures, such as those in Edgington and Alphey (2018) and Khamis *et al.* (2018, 2020).

12.6 Linking Theory and Experimentation

The development of gene drive technologies, including UD, has generally followed a design–build–test cycle (Fig. 12.6). At each stage, modelling can play an important role in designing, understanding and analysing experimental work. Thus, in addition to providing insights, modelling can save a significant amount of research time, effort and
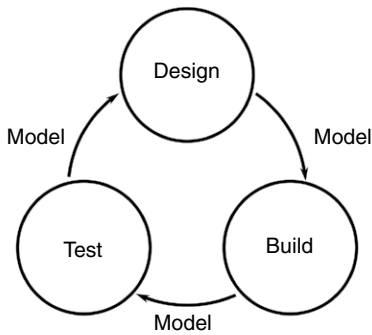
Fig. 12.6. A cartoon showing the design–build–test cycle commonly followed in the development of gene drive technologies and that mathematical modelling is an important tool within each phase.

money. To date there has been relatively little published experimental work on UD and so this section focuses on how one could link theory and experiment when experimental data becomes available. This will partially be informed by studies on alternative gene drive classes and will describe each phase of the design–build–test cycle; however, since the 'design–build' aspect of this cycle has largely been covered in previous sections, it will not be discussed further here.

In the 'build–test' phase, experimentation commonly focuses on discrete generation laboratory cage-based experiments. Models of the form shown in section 12.4 assume discrete generations and so they are ideal for predicting and analysing experiments of that form. In the first instance, specific test crosses between transgenic insect strains can be performed and the number of offspring of each resulting genotype counted/screened (as in Hammond *et al.,* 2016, 2017, 2020; Kyrou *et al.*, 2018; Adolfi *et al.*, 2020; Champer *et al.,* 2020b,c; Simoni *et al.*, 2020), with fluorescent markers commonly used to distinguish between types. This allows a first approximation of various system parameters (for example, relative fitness, toxin penetrance and strength of the antidote effect (Buchman *et al.*, 2018b; Webster *et al.*, 2020)) to be generated by calculating ratios between the mean number of each genotype produced. These can then be used to parameterize models and predict the expected outcomes of gene drive cage trials.

Note that other gene drive classes may approximate fitness costs by fitting models to data from cage trials with only a single transgenic construct present (e.g. Webster *et al.*, 2020), but this is not feasible for UD where a single transgenic construct produces a lethal phenotype.

Following the 'test' phase, i.e., after laboratory cage trial experiments, prior predictions of gene drive behaviour can be compared with the actual cage trial data. Using the same mathematical models (and potentially stochastic versions also, especially given the relatively small numbers of individual mosquitoes typical of a laboratory cage experiment), one can then assess whether model predictions are consistent with the observed outcomes (as in Hammond *et al.*, 2016, 2020; Buchman *et al.*, 2018b; Kyrou *et al.*, 2018; Pham *et al.*, 2019; Adolfi *et al.*, 2020; Champer *et al.*, 2020c; Simoni *et al.*, 2020; Webster *et al.*, 2020). If model and experimental results are consistent, then parameters can be varied to identify potential areas for improvement in the gene drive design. Conversely, if model and experimentation are not consistent, then further modelling may be required to identify sources of this mismatch, potentially informing future models and experimental designs (as in Hammond *et al.*, 2017, 2020, for CRISPR-based gene drives).

12.7 Alternative Configurations of UD

Previous sections focused on a specific configuration of UD, namely that based on two mutually repressing bi-sex toxin genes, inserted into two unlinked and independently segregating genomic loci. Similarly, we have primarily focused on the release of both transgenic males and females. However, there is a wide range of alternative methods for engineering/releasing UD systems, a variety of which have been studied previously.

For the UD design considered thus far, a potential variation is in the time at which the toxin takes effect. This has been studied for a UD system with toxins and transgene fitness costs acting either in early (before

density-dependent competition, for example eggs or early instar larvae for mosquitoes) or late (following density-dependent competition, for example pupae or pharate adults in mosquitoes) developmental stages (Edgington and Alphey, 2018; Khamis et al., 2018). While early- or late-acting lethal/fitness effects do not have any impact on threshold introduction frequencies, they can have more impact on other traits. For instance, Khamis et al. (2018) found that, for a system spreading a cargo gene conferring refractoriness to a pathogen, late-acting lethality produced a slightly larger reduction in disease burden. This is likely due to the greater reduction in both equilibrium and (transiently attained) minimum population sizes observed with late-acting lethality (as seen in Edgington and Alphey, 2018). Despite this potential epidemiological benefit, a greater reduction in population size may not always be good news. For example, Ae. aegypti mosquitoes are known to compete with Aedes albopictus (Edgerly et al., 1993; Juliano et al., 2002; Armistead et al., 2008). Therefore, an Ae. aegypti population may be displaced during the period in which the population is reduced by a late-acting UD – potentially reducing the epidemiological benefit as Ae. albopictus are also competent vectors of a similar set of pathogens, including dengue viruses (WHO, 2011). This necessitates some knowledge of ecological factors in the vicinity of gene drive target areas, and could be addressed by modelling approaches similar to those used for sterile insect technique (SIT) and release of insects carrying a dominant lethal (RIDL)-based control (Bonsall et al., 2010).

Other possible UD configurations revolve around the use of sex-specific toxins or insect releases, rather than the bi-sex versions considered above. Such considerations have been studied in terms of their effect on release thresholds and degrees of tolerable transgene fitness costs (Edgington and Alphey, 2017). These results showed that considering either male-only release(s) of gene drive-carrying individuals or female-specific toxins results in a lesser ability to tolerate fitness costs and higher introduction thresholds.

In a two-locus UD configuration, it is possible that the suppressor element from one transgenic construct is not sufficient to inactivate two copies of the toxin gene from the other transgenic construct. In the context of UD, this has been referred to as 'weak suppression' (Edgington and Alphey, 2017, 2018); however, the mathematical models and predicted dynamics are equally applicable to systems based on reciprocal chromosome translocations (Buchman et al., 2018b). These studies showed that reciprocal chromosome translocations (or weakly suppressed UD) generally have a higher introduction threshold than the UD systems discussed here. As discussed previously, this represents a trade-off between the increased cost/difficulty of gene drive introgression and the increased reliability of gene drive confinement to the target population.

Several gene drive concepts are based on toxin–antidote systems. The UD system considered thus far assumes two mutually suppressing lethals, each of which comprise a 'toxin' gene and an antidote that suppresses its effect, perhaps RNAi targeting the toxin gene. Other toxin–antidote concepts can also provide threshold-dependent gene drives. For example, a synthetic Medea drive was constructed in Drosophila using a maternally contributed (RNAi) toxin with zygotic expression of the antidote only in those offspring inheriting the Medea element (Buchman et al., 2018a). Medea is a low-threshold drive, zero-threshold in the absence of fitness costs, but a mutually repressing pair of such elements can provide a threshold-dependent drive known as UD[MEL] (Akbari et al., 2013) or double Medea (Wimmer, 2013). For a rather different molecular basis, a CRISPR/Cas9 system (toxin) can be used to disrupt an essential endogenous gene, which can then be rescued with a recoded – and therefore toxin-resistant – antidote. A range of threshold-dependent gene drives using this technology have previously been modelled (Champer et al., 2021) and are based on the use of cleave and rescue (Oberhofer et al., 2019) or CRISPR toxin–antidote (Champer et al., 2021; Champer et al., 2020a; Champer et al., 2020b) elements. These have been discussed extensively

in the original sources and produce broadly similar behaviour to the approach(es) discussed here. Additionally, the mathematical modelling frameworks considered in the studies listed above are similar to those explored throughout this chapter and so we do not discuss results of these studies any further.

12.8 Areas of Future Interest

Despite all the modelling work discussed above, there remain several areas in which further modelling could elucidate various characteristics of UD gene drives. Some have briefly been mentioned in the relevant sections above and so we focus predominantly on areas not yet discussed.

Above, we discussed the use of laboratory cage trial experiments for inferring parameters of the UD system. While these are useful for predicting system performance, these estimates are inherently flawed when moving into the field since they assume that laboratory wild-type strains – and environments – are a good approximation of insects in the wild. In practice, laboratory wild-type strains are recognized as having lower fitness than their wild counterparts (Leftwich *et al.*, 2021), likely due to many generations of laboratory adaptation (Leftwich *et al.*, 2016; Ross *et al.*, 2019). Models can capture the impact of this to a certain degree by considering variation of relative fitness parameters about laboratory-derived estimates. However, the transition toward field-based experiments will potentially necessitate more detailed models capturing a range of ecological, behavioural and fitness effects (some of which have been discussed above). This enhanced modelling can then help to inform the design of UD releases as they progress from small-scale field cage trials right up to the eventual release in full large-scale control programmes.

A feature of most of the modelling discussed here is that it is deterministic and so does not account for the stochasticity inherent in the real world. In the context of UD, this will be important when the release of transgenic insects results in a gene drive frequency close to (or even below) the introduction threshold calculated from deterministic mathematical models (i.e., the unstable equilibrium discussed above). Here stochastic models can provide insight into the expected likelihood of success or failure (i.e., the probability that a UD system increases or decreases in frequency) of a given release strategy. To date, stochastic modelling of UD systems has been limited, to our knowledge, to only Marshall and Hay (2012) for this UD configuration. However, some stochastic modelling frameworks have been used to study other gene drive classes, from which such work could take a lead (for example: Magori *et al.*, 2009; Champer *et al.*, 2020a; Edgington *et al.*, 2020a,b; Sánchez *et al.*, 2020a; Wu *et al.*, 2021).

A common feature in the modelling of many gene drive classes, and, in particular, toxin–antidote-based approaches, is an assumption that toxins and antidotes are fully penetrant (i.e., that toxins kill 100% of target genotypes and antidotes rescue 100% of carriers to full fitness). However, gene drive components engineered in the laboratory may not give this degree of efficacy. Laboratory experiments, for example life history analysis of different genotypes, can provide initial estimates of such incomplete penetrance. These data could be incorporated into models similar to those in section 12.4. In the absence of working gene drive components to test in the laboratory, one can use the same model to explore the expected behaviour for a range of toxin and antidote penetrance parameters, thus setting performance targets for laboratory-engineered gene drive components. This could be used to assess performance metrics including threshold frequencies, the speed of spread, the system invasiveness (with *n*-deme versions of the models) and tolerable fitness costs – all of which are likely to be vital when transitioning from laboratory to field-based testing.

The motivation for genetic control of mosquitoes is to reduce or prevent morbidity and mortality from mosquito-borne diseases. Thus, it is important to explore the anticipated epidemiological impact(s) expected from a given gene drive and release

strategy. This can be explored by incorporating a gene drive model into a standard epidemiological modelling framework, for example susceptible–exposed–infectious–recovered (S-E-I-R) or a variety of extensions/modifications as previously considered for *Wolbachia* (Ndii *et al.*, 2015, 2016a,b; Zhang and Lui, 2020) or RIDL (Atkinson *et al.*, 2007) control approaches in *Ae. aegypti* mosquitoes. This will likely require the use of a population dynamics model similar to those of Khamis *et al.* (2018) and Edgington and Alphey (2018) for two main reasons: (i) models must produce results for gene drive and epidemiological dynamics at all time points; and (ii) the respective sizes of human and insect (vector) populations are important in determining a pathogen's force of infection. Such models can provide important insights into potential epidemiological impacts. However, various factors required to formulate these models (such as infection numbers, exact population sizes, transmissibility of pathogen(s) and biting frequency) can be extremely difficult to measure, meaning that the consideration of model uncertainty will be important when interpreting results.

This chapter has focused on the use of mathematical modelling to predict the efficacy of UD gene drives from molecular design and laboratory testing right through to field testing and final applications. Such studies will likely be important in providing an evidential basis upon which regulatory decisions can be made. As further laboratory-based testing provides more and higher-quality data, we would anticipate that more detailed and species-specific models will be developed, providing greater insight into the anticipated efficacy of UD systems. Likewise, as more field studies into the ecology of potential gene drive target species and field-trial releases (of this or other technologies) become available, more detailed ecological, epidemiological and behavioural factors can be studied and incorporated into models, enabling the best possible predictions of gene drive function following release of transgenic insects. The previous literature and future focus areas discussed here demonstrate the key role that modelling plays in the development of gene drive technologies and emphasizes the necessity for gene drive research and development to follow an interdisciplinary approach. This will ensure that any future gene drive release has the greatest opportunity to function as intended, thus providing the maximum possible beneficial impact.

Acknowledgements

References

Adolfi, A., Gantz, V.M., Jasinskiene, N., Lee, H.-F., Hwang, K. *et al.* (2020) Efficient population modification gene-drive rescue system in the malaria mosquito *Anopheles stephensi*. *Nature Communications* 11, 5553.

Akbari, O.S., Matzen, K.D., Marshall, J.M., Huang, H., Ward, C.M. and Hay, B.A. (2013) A synthetic gene drive system for local, reversible modification and suppression of insect populations. *Current Biology* 23, 671–677.

Alphey, L. (2014) Genetic control of mosquitoes. *Annual Review of Entomology* 59, 205–224.

Alphey, L.S., Crisanti, A., Randazzo, F. and Akbari, O.S. (2020) Opinion: Standardizing the definition of gene drive. *Proceedings of the National Academy of Sciences USA* 117, 30864–30867.

Alphey, N. and Bonsall, M.B. (2014) Interplay of population genetics and dynamics in the genetic control of mosquitoes. *Journal of The Royal Society Interface* 11, 20131071.

Altrock, P.M., Traulsen, A. and Reed, F.A. (2011) Stability properties of underdominance in finite subdivided populations. *PLoS Computational Biology* 7, e1002260.

Altrock, P.M., Traulsen, A., Reeves, R.G. and Reed, F.A. (2010) Using underdominance to bi-stably transform local populations. *Journal of Theoretical Biology* 267, 62–75.

Armistead, J.S., Arias, J.R., Nishimura, N. and Lounibos, L.P. (2008) Interspecific larval competition between *Aedes albopictus* and *Aedes japonicus* (Diptera: Culicidae) in northern Virginia. *Journal of Medical Entomology* 45, 629–637.

Atkinson, M.P., Su, Z., Alphey, N., Alphey, L.S., Coleman, P.G. and Wein, L.M. (2007) Analyzing the control of mosquito-borne diseases by a dominant lethal genetic system. *Proceedings of the National Academy of Sciences USA* 104, 9540–9545.

Beaghton, A., Beaghton, P.J. and Burt, A. (2016) Gene drive through a landscape: reaction–diffusion models of population suppression and elimination by a sex ratio distorter. *Theoretical Population Biology* 108, 51–69.

Bellows, T.S. (1981) The descriptive properties of some models for density dependence. *Journal of Animal Ecology* 50, 139–156.

Bonsall, M.B., Yakob, L., Alphey, N. and Alphey, L. (2010) Transgenic control of vectors: the effects of interspecific interactions. *Israel Journal of Ecology and Evolution* 56, 353–370.

Buchman, A., Marshall, J.M., Ostrovski, D., Yang, T. and Akbari, O.S. (2018a) Synthetically engineered *Medea* gene drive system in the worldwide crop pest *Drosophila suzukii*. *Proceedings of the National Academy of Sciences USA* 115, 4725–4730.

Buchman, A.B., Ivy, T., Marshall, J.M., Akbari, O.S. and Hay, B.A. (2018b) Engineered reciprocal chromosome translocations drive high threshold, reversible population replacement in *Drosophila*. *ACS Synthetic Biology* 7, 1359–1370.

Buchman, A., Gamez, S., Li, M., Antoshechkin, I., Li, H.-H. *et al.* (2019) Engineered resistance to Zika virus in transgenic *Aedes aegypti* expressing a polycistronic cluster of synthetic small RNAs. *Proceedings of the National Academy of Sciences USA* 116, 3656–3661.

Buchman, A., Shriner, I., Yang, T., Liu, J., Antoshechkin, I. *et al.* (2021) Engineered reproductively isolated species drive reversible population replacement. *Nature Communications* 12, 3281.

Champer, J., Buchman, A. and Akbari, O.S. (2016) Cheating evolution: engineering gene drives to manipulate the fate of wild populations. *Nature Reviews Genetics* 17, 146–159.

Champer, J., Reeves, R., Oh, S.Y., Liu, C., Liu, J. *et al.* (2017) Novel CRISPR/Cas9 gene drive constructs reveal insights into mechanisms of resistance allele formation and drive efficiency in genetically diverse populations. *PLoS Genetics* 13, e1006796.

Champer, J., Kim, I.K., Champer, S.E., Clark, A.G. and Messer, P.W. (2020a) Performance analysis of novel toxin-antidote CRISPR gene drive systems. *BMC Biology* 18, 27.

Champer, J., Lee, E., Yang, E., Liu, C., Clark, A.G. and Messer, P.W. (2020b) A toxin–antidote CRISPR gene drive system for regional population modification. *Nature Communications* 11, 1082.

Champer, J., Yang, E., Lee, E., Liu, J., Clark, A.G. and Messer, P.W. (2020c) A CRISPR homing gene drive targeting a haplolethal gene removes resistance alleles and successfully spreads through a cage population. *Proceedings of the National Academy of Sciences USA* 117, 24377–24383.

Champer, J., Zhao, J., Champer, S.E., Liu, J. and Messer, P.W. (2020d) Population dynamics of underdominance gene drive systems in continuous space. *ACS Synthetic Biology* 9, 779–792.

Champer, J., Champer, S.E., Kim, I.K., Clark, A.G. and Messer, P.W. (2021) Design and analysis of CRISPR-based underdominance toxin-antidote gene drives. *Evolutionary Applications* 14, 1052–1069.

Curtis, C. (1968) Possible use of translocations to fix desirable genes in insect pest populations. *Nature* 218, 368–369.

Davis, S., Bax, N. and Grewe, P. (2001) Engineered underdominance allows efficient and economical introgression of traits into pest populations. *Journal of Theoretical Biology* 212, 83–98.

Dhole, S., Vella, M.R., Lloyd, A.L. and Gould, F. (2018) Invasion and migration of spatially self-limiting gene drives: a comparative analysis. *Evolutionary Applications* 11, 794–808.

Dhole, S., Lloyd, A.L. and Gould, F. (2019) Tethered homing gene drives: a new design for spatially restricted population replacement and suppression. *Evolutionary Applications* 12, 1688–1702.

Dhole, S., Lloyd, A.L. and Gould, F. (2020) Gene drive dynamics in natural populations: the importance of density dependence, space, and sex. *Annual Review of Ecology, Evolution, and Systematics* 51, 505–531.

Edgerly, J.S., Willey, M.S. and Livdahl, T.P. (1993) The community ecology of *Aedes* egg hatching: implications for a mosquito invasion. *Ecological Entomology* 18, 123–128.

Edgington, M.P. and Alphey, L.S. (2017) Conditions for success of engineered underdominance gene drive systems. *Journal of Theoretical Biology* 430, 128–140.

Edgington, M.P. and Alphey, L.S. (2018) Population dynamics of engineered underdominance and killer-rescue gene drives in the control of disease vectors. *PLoS Computational Biology* 14, e1006059.

Edgington, M.P. and Alphey, L.S. (2019) Modeling the mutation and reversal of engineered underdominance gene drives. *Journal of Theoretical Biology* 479, 14–21.

Edgington, M.P., Harvey-Samuel, T. and Alphey, L. (2020a) Population-level multiplexing: a promising strategy to manage the evolution of resistance against gene drives targeting a neutral locus. *Evolutionary Aplications* 13, 1939–1948.

Edgington, M.P., Harvey-Samuel, T. and Alphey, L. (2020b) Split drive killer–rescue provides a novel threshold-dependent gene drive. *Scientific Reports* 10, 20520.

Ermentrout, B. (2002) *Simulating, Analyzing, and Animating Dynamical Systems: A Guide to XPPAUT for Researchers and Students*. SIAM, Philadelphia, Pennsylvania.

Franz, A.W., Sanchez-Vargas, I., Adelman, Z.N., Blair, C.D., Beaty, B.J. *et al.* (2006) Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. *Proceedings of the National Academy of Sciences USA* 103, 4198–4203.

Franz, A.W., Sanchez-Vargas, I., Raban, R.R., Black IV, W.C., James, A.A. and Olson, K.E. (2014) Fitness impact and stability of a transgene conferring resistance to dengue-2 virus following introgression into a genetically diverse *Aedes aegypti* strain. *PLoS Neglected Tropical Diseases* 8, e2833.

Haag-Liautard, C., Dorris, M., Maside, X., Macaskill, S., Halligan, D.L. *et al.* (2007) Direct estimation of per nucleotide and genomic deleterious mutation rates in *Drosophila*. *Nature* 445, 82–85.

Haller, B.C. and Messer, P.W. (2016) SLiM 2: flexible, interactive forward genetic simulations. *Molecular Biology and Evolution* 34, 230–240.

Haller, B.C. and Messer, P.W. (2019) SLiM 3: forward genetic simulations beyond the Wright–Fisher Model. *Molecular Biology and Evolution* 36, 632–637.

Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C. *et al.* (2016) A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nature Biotechnology* 34, 78–83.

Hammond, A.M., Kyrou, K., Bruttini, M., North, A., Galizi, R. *et al.* (2017) The creation and selection of mutations resistant to a gene drive over multiple generations in the malaria mosquito. *PLoS Genetics* 13, e1007039.

Hammond, A., Karlsson, X., Morianou, I., Kyrou, K., Beaghton, A. *et al.* (2020) Regulation of gene drive expression increases invasive potential and mitigates resistance. *bioRxiv* 360339.

Harvey-Samuel, T., Campbell, K., Edgington, M.P. and Alphey, L. (2019) Trialling gene drives to control invasive species: what, where and how? In Veitch, C.R., Clout, M.N., Martin, A.R., Russell, J.C. and West C.J. (eds) *Island Invasives: Scaling Up to Meet the Challenge*. Occasional Paper SSC no. 662. IUCN, Gland, Switzerland, pp. 618–627.

Huang, Y., Lloyd, A.L., Legros, M. and Gould, F. (2011) Gene-drive into insect populations with age and spatial structure: a theoretical assessment. *Evolutionary Applications* 4, 415–428.

James, A.A. (2005) Gene drive systems in mosquitoes: rules of the road. *Trends in Parasitology* 21, 64–67.

Juliano, S.A., O'Meara, G.F., Morrill, J.R. and Cutwa, M.M. (2002) Desiccation and thermal tolerance of eggs and the coexistence of competing mosquitoes. *Oecologia* 130, 458–469.

Keightley, P.D., Ness, R.W., Halligan, D.L. and Haddrill, P.R. (2014) Estimation of the spontaneous mutation rate per nucleotide site in a *Drosophila melanogaster* full-sib family. *Genetics* 196, 313–320.

Khamis, D., El Mouden, C., Kura, K. and Bonsall, M.B. (2018) Ecological effects on underdominance threshold drives for vector control. *Journal of Theoretical Biology* 456, 1–15.

Khamis, D., El Mouden, C., Kura, K. and Bonsall, M. (2020) The effect of dispersal and preferential mating on the genetic control of mosquitoes. *bioRxiv* 114413.

Kyrou, K., Hammond, A.M., Galizi, R., Kranjc, N., Burt, A. *et al.* (2018) A CRISPR–Cas9 gene drive targeting doublesex causes complete population suppression in caged *Anopheles gambiae* mosquitoes. *Nature Biotechnology* 36(11), 1062–1066.

Lanzaro, G.C., Campos, M., Crepeau, M., Cornel, A., Estrada, A. *et al.* (2021) Selection of sites for field trials of genetically engineered mosquitoes with gene drive. *Evolutionary Applications* 14, 2147–2161.

Leftwich, P.T., Bolton, M. and Chapman, T. (2016) Evolutionary biology and genetic techniques for insect control. *Evolutionary Applications* 9, 212–230.

Leftwich, P.T., Edgington, M.P., Harvey-Samuel, T., Carabajal Paladino, L.Z., Norman, V.C. and Alphey, L. (2018) Recent advances in threshold-dependent gene drives for mosquitoes. *Biochemical Society Transactions* 46, 1203–1212.

Leftwich, P.T., Spurgin, L.G., Harvey-Samuel, T., Thomas, C.J.E., Paladino, L.C. *et al.* (2021) Genetic pest management and the background genetics of release strains. *Philosophical Transactions of the Royal Society B: Biological Sciences* 376, 20190805.

Long, K.C., Alphey, L., Annas, G.J., Bloss, C.S., Campbell, K.J. *et al.* (2020) Core commitments for field trials of gene drive organisms. *Science* 370(6523), 1417–1419.

Magori, K. and Gould, F. (2006) Genetically engineered underdominance for manipulation of pest populations: a deterministic model. *Genetics*, 172(4), 2613–2620.

Magori, K., Legros, M., Puente, M.E., Focks, D.A., Scott, T.W., Lloyd, A.L. and Gould, F. (2009) Skeeter Buster: a stochastic, spatially explicit modeling tool for studying *Aedes aegypti* population replacement and population suppression strategies. *PLoS Neglected Tropical Diseases* 3, e508.

Marshall, J.M. and Hay, B.A. (2012) Confinement of gene drive systems to local populations: a comparative analysis. *Journal of Theoretical Biology* 294, 153–171.

Maselko, M., Feltman, N., Upadhyay, A., Hayward, A., Das, S. *et al.* (2020) Engineering multiple species-like genetic incompatibilities in insects. *Nature Communications* 11(1), 4468.

Maynard Smith, J. and Slatkin, M. (1973) The stability of predator–prey systems. *Ecology* 54, 384–391.

NASEM (National Academies of Sciences, Engineering, and Medicine) (2016) *Gene Drives on the Horizon: Advancing Science, Navigating Uncertainty, and Aligning Research with Public Values*. The National Academies Press, Washington, DC.

Ndii, M.Z., Hickson, R.I., Allingham, D. and Mercer, G.N. (2015) Modelling the transmission dynamics of dengue in the presence of *Wolbachia*. *Mathematical Biosciences* 262, 157–166.

Ndii, M.Z., Allingham, D., Hickson, R.I. and Glass, K. (2016a) The effect of *Wolbachia* on dengue dynamics in the presence of two serotypes of dengue: symmetric and asymmetric epidemiological characteristics. *Epidemiology and Infection* 144, 2874–2882.

Ndii, M. Z., Allingham, D., Hickson, R.I. and Glass, K. (2016b) The effect of *Wolbachia* on dengue outbreaks when dengue is repeatedly introduced. *Theoretical Population Biology* 111, 9–15.

Oberhofer, G., Ivy, T. and Hay, B.A. (2019) Cleave and Rescue, a novel selfish genetic element and general strategy for gene drive. *Proceedings of the National Academy of Sciences USA* 116(13), 6250–6259.

Pham, T.B., Phong, C.H., Bennett, J.B., Hwang, K., Jasinskiene, N. *et al.* (2019) Experimental population modification of the malaria vector mosquito, *Anopheles stephensi. PLoS Genetics* 15, e1008440.

Reeves, R.G., Bryk, J., Altrock, P.M., Denton, J.A. and Reed, F.A. (2014) First steps towards underdominant genetic transformation of insect populations. *PLoS ONE* 9, e97557.

Ross, P.A., Endersby-Harshman, N.M. and Hoffmann, A.A. (2019) A comprehensive assessment of inbreeding and laboratory adaptation in *Aedes aegypti* mosquitoes. *Evolutionary Applications* 12(3), 572–586.

Sánchez Castellanos, H.M., Bennett, J.B., Wu, S.L., Rašić, G., Akbari, O.S. and Marshall, J.M. (2020a) Modeling confinement and reversibility of threshold-dependent gene drive systems in spatially-explicit *Aedes aegypti* populations. *BMC Biology* 18(1), 50.

Sánchez Castellanos, H.M., Wu, S.L., Bennett, J.B. and Marshall, J.M. (2020b) MGDrivE: A modular simulation framework for the spread of gene drives through spatially explicit mosquito populations. *Methods in Ecology and Evolution* 11, 229–239.

Simoni, A., Hammond, A.M., Beaghton, A.K., Galizi, R., Taxiarchi, C. *et al.* (2020) A male-biased sex-distorter gene drive for the human malaria vector *Anopheles gambiae*. *Nature biotechnology* 38(9), 1054–1060.

Sinkins, S.P. and Gould, F. (2006) Gene drive systems for insect disease vectors. *Nature Reviews Genetics* 7(6), 427–435.

Tanaka, H., Stone, H.A. and Nelson, D.R. (2017) Spatial gene drives and pushed genetic waves. *Proceedings of the National Academy of Sciences USA* 114, 8452–8457.

Webster, S.H., Vella, M.R. and Scott, M.J. (2020) Development and testing of a novel killer rescue self-limiting gene drive system in *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences* 287, 20192994.

Wilson, D.S. and Turelli, M. (1986) Stable underdominance and the evolutionary invasion of empty niches. *The American Naturalist* 127, 835–850.

Wimmer, E.A. (2013) Insect biotechnology: controllable replacement of disease vectors. *Current Biology* 23(10), R453-R456.

Windbichler, N., Menichelli, M., Papathanos, P.A., Thyme, S.B., Li, H. *et al.* (2011) A synthetic homing endonuclease-based gene drive system in the human malaria mosquito. *Nature* 473(7346), 212–215.

WHO (2011) *Comprehensive Guideline for Prevention and Control of Dengue and Dengue Haemorrhagic Fever*. Revised and expanded edition. World Health Organization Regional Office for South-East Asia, New Delhi, India.

Wu, S.L., Bennett, J.B., Sánchez Castellanos, H.M., Dolgert, A.J., León, T.M. and Marshall, J.M. (2021) MGDrivE 2: A simulation framework for gene drive systems incorporating seasonality and epidemiological dynamics. *PLoS Computational Biology* 17, e1009030.

Zhang, H. and Lui, R. (2020) Releasing *Wolbachia*-infected *Aedes aegypti* to prevent the spread of dengue virus: a mathematical study. *Infectious Disease Modelling* 5, 142–160.

13 Tsetse Paratransgenesis: a Novel Strategy for Reducing the Spread of African Trypanosomiases

Brian L. Weiss*, Liu Yang and Serap Aksoy

Department of Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, Connecticut

---

13.1 Tsetse as Vectors of Parasitic African Trypanosomes

Tsetse flies (Diptera: Glossinidae) are the sole vectors of cyclical pathogenic trypanosomes in sub-Saharan Africa. Human African trypanosomiasis (HAT), or sleeping sickness, is a zoonosis caused by the flagellated protozoa *Trypanosoma brucei rhodesiense* in East and southern Africa and *Trypanosoma brucei gambiense* in West and central Africa. Following the epidemics that killed hundreds of thousands of people in the 20th century, the World Health Organization (WHO) roadmap to control neglected tropical diseases (NTDs) includes HAT elimination by 2030 (Simarro *et al.*, 2011; Barrett, 2018). However, elimination of HAT will require continued efforts to tackle a range of expected and unexpected challenges given that significant disparities exist on the continent, with little reliable data being provided by countries with conflict, and from the remote regions where HAT is typically transmitted (Franco *et al.*, 2020). In addition to HAT, animal African trypanosomiasis (AAT) is rampant throughout sub-Saharan Africa where the disease severely impedes agricultural development, thus restricting nutritional sources and economic prosperity in tsetse-infested areas.

Trypanosome transmission requires interaction between several organisms, including the pathogen, the insect vector and vertebrate hosts. While this life cycle can be complicated, it also provides multiple opportunities for controlling disease. Unfortunately, mammalian vaccines, which are important tools for controlling infection, are currently unavailable. The parasite is protected from antibody-mediated lysis in the mammalian host due to its ability to modify its surface coat via a process of antigenic variation, which has hindered the development of mammalian vaccines (Vickerman, 1978). Thus, the control of *gambiense* disease relies on active surveillance followed by treatment of the identified infected hosts. In this regard, significant progress has been achieved in recent years on the development of effective therapeutics (Ndung'u *et al.*, 2020) but, as case numbers decline, active surveillance becomes logistically difficult and costly and necessitates supplementation by vector control (Lehane *et al.*, 2016; Aksoy *et al.*, 2017; Mahamat *et al.*, 2017). Control of *rhodesiense* disease is further

---

*Corresponding author, email: brian.weiss@yale.edu

complicated by the fact that *T. b. rhodesiense* has multiple domestic and wild animal reservoirs, which provide abundant sources of parasites for transmission by tsetse. Hence, vector control is necessary and, if consistently applied, can be highly effective to break the transmission cycle for *rhodesiense* disease. However, in vast and remote areas where disease is most prevalent, long-term sustainability of the existing vector control tools has been unsatisfactory and has led to a rebound of fly populations.

While vector control remains necessary and effective, each method currently in use, such as trapping and insecticides, presents limitations because of their requirement for extensive community participation and undesirable environmental consequences. Recently, small target traps have been employed in HAT control programmes and found to be successful in reducing vector populations (Rayaisse *et al.*, 2011; Esterhuizen *et al.*, 2011; Solano *et al.*, 2013; Vale *et al.*, 2015; Mahamat *et al.*, 2017; Ndung'u *et al.*, 2020). In addition, the use of an area-wide control strategy that integrates the sterile insect technique (SIT) is effective for tsetse control, and following a successful initial trial on the island of Zanzibar, its application on the mainland has been pursued (Vreysen *et al.*, 2000; Abd-Alla *et al.*, 2013). Their reliance on extensive community acceptance for sustainable implementation is a major drawback, especially when disease prevalence is low. Another criticism of tsetse SIT programmes is that the large number of released sterile male flies, which, like females, feed exclusively on vertebrate blood, have the potential to transmit disease-causing parasites. Recent advances in biotechnology have the potential to enhance the efficacy of SIT by diminishing the vectorial capacity of the tsetse colony used for the SIT releases (Aksoy *et al.*, 2001; Rayaisse *et al.*, 2011; Aksoy *et al.*, 2017; Diall *et al.*, 2017). Here we review progress on one such method, called paratransgenesis. This control strategy alters tsetse's gut environment so that it becomes inhospitable to processes involved in the establishment of trypanosome infections. Paratransgenesis relies on the use of genetically modified commensal endosymbionts

that reside naturally in tsetse's midgut (Beard *et al.*, 1993a; Aksoy *et al.*, 2008; De Vooght *et al.*, 2012, 2014, 2018; Rio *et al.*, 2004; Yang *et al.*, 2021), or on the recolonization of tsetse's midgut with exogenous commensals or other closely related bacteria (Weiss *et al.*, 2019; Medina Munoz *et al.*, 2020) that confer antitrypanosomal traits. In addition to being applied to SIT programmes, paratransgenic parasite-resistant tsetse flies can be used in population replacement strategies, which have the advantage of requiring minimal community participation in comparison with other methods.

13.2 Tsetse Reproduction and Symbiosis

13.2.1 Tsetse reproduction

Most insects reproduce oviparously, during which females lay clutches of fertilized eggs in their environment. In contrast, tsetse utilizes a unique mode of reproduction called adenotrophic viviparity. In this case female flies ovulate one oocyte per gonotrophic cycle, and the subsequent embryo and all larval stages develop in the female's uterus (Attardo *et al.*, 2020). Female tsetse give birth to a well-developed 3rd-instar larva that immediately pupates. Tsetse's gonotrophic cycle is complete when an adult fly ecloses from its pupal case 30 days later. During intra-uterine larval development, tsetse progeny receive nourishment via secretions from the modified maternal accessory gland (designated the 'milk gland'). These secretions contain proteins, lipids, amino acids, immuno-modulatory molecules and symbiotic bacteria (Benoit *et al.*, 2015; Wang and Aksoy, 2012; Attardo *et al.*, 2008).

13.2.2 Tsetse's endogenous endosymbionts

Much of our knowledge about the functional aspects of tsetse symbiosis has been acquired using laboratory-reared *Glossina morsitans*

morsitans, which house a simple, maternally transmitted microbiota composed predominantly of obligate *Wigglesworthia glossinidia*, commensal *Sodalis glossinidius* and parasitic *Wolbachia* and *Spiroplasma*. Two distinct populations of *Wigglesworthia* can be found in adult tsetse flies. One of these populations resides intracellularly within bacteriocytes, which collectively comprise an organ called the bacteriome that is found immediately adjacent to tsetse's anterior midgut (Aksoy, 1995, 2000). The second population of *Wigglesworthia* is found extracellularly in maternal milk within the female accessory glands (Attardo *et al*., 2008). The bacteria in the milk are transmitted from the mother to her developing intra-uterine larvae. Phylogenetic analysis of *Wigglesworthia* from different tsetse species shows concordant history with their host. This finding suggests that a tsetse ancestor had been infected with a bacterium some 50–80 million years ago, and from this ancestral pair extant species of tsetse and associated *Wigglesworthia* strains radiated without horizontal transfer events between species (Chen *et al*., 1999). The ancient association between tsetse and *Wigglesworthia* is further evidenced by the fact that this bacterium's chromosome (analysed from two distinct tsetse host species) has undergone a drastic size reduction (to about 700 kb in size), exhibits exceptional A-T bias (82%) and contains no transposons or phage-related elements (Akman *et al*., 2002; Rio *et al*., 2012). The *Wigglesworthia* proteome is also highly streamlined and is composed of only 620 proteins. Interestingly, despite this reduction, *Wigglesworthia*'s chromosome encodes several complete vitamin biosynthesis pathways, and *Wigglesworthia* supplemented B-vitamins, which are present at low concentrations in its vertebrate blood-specific diet, are essential for maintaining tsetse's fecundity (Pais *et al*., 2008; Snyder *et al*., 2010; Michalkova *et al*., 2014; Snyder and Rio, 2015). In addition to its role in host nutrient provisioning, *Wigglesworthia* is involved in the development and function of tsetse's immune system. More specifically, flies that undergo larval development in the absence of *Wigglesworthia* fail to produce immunocompetent haemocytes, exhibit dysfunctional expression of genes that encode anti-parasitic effector molecules and fail to secrete a structurally robust midgut lining peritrophic matrix (PM) barrier (Weiss and Aksoy, 2011; Weiss *et al*., 2011, 2012; Benoit *et al*., 2017). The PM acts as a physical barrier that lines tsetse's midgut and protects the fly from infection with pathogenic microorganisms, including African trypanosomes (Hegedus *et al*., 2009; Aksoy, 2019; Erlandson *et al*., 2019). Taken together, these findings are indicative of the steadfast association between tsetse and its obligate symbiont.

The second tsetse symbiont (also maternally transmitted) is the facultative commensal *Sodalis glossinidius* (Dale and Maudlin, 1999). *Sodalis* infection prevalence and density vary within field-captured tsetse (Mbewe *et al*., 2015; Griffith *et al*., 2018). Unlike *Wigglesworthia*, *Sodalis* exhibits a broad host tissue tropism and can be found both inter- and intracellularly in the midgut, muscle, fat body, milk gland and salivary glands (Cheng and Aksoy, 1999; Balmand *et al*., 2013). The association of *Sodalis* with tsetse is more recent compared with *Wigglesworthia*. In fact, phylogenetic analysis of *Sodalis* isolated from distantly related tsetse host species shows little differentiation between these symbionts. These findings suggest that distinct tsetse species likely acquired *Sodalis* independently via multiple horizontal transfer events (Aksoy *et al*., 1997; Weiss *et al*., 2006). Analysis of the *Sodalis* 4.2 megabase chromosome indicates that this bacterium is closely related to several free-living enterics (including *Escherichia coli*, *Salmonella* and *Yersinia*) in regard to chromosomal size, gene content and gene synteny (Toh *et al*., 2006). The high number (972) of pseudogenes present on the chromosome, together with an exceptionally low protein coding capacity (49%), indicates that *Sodalis* is evolving away from a free-living existence and towards a true symbiotic one.

The functional contribution of *Sodalis* to tsetse's physiological well-being is unclear. However, in field-captured tsetse, a positive correlation exists between *Sodalis* prevalence and density, and trypanosome infection prevalence (Farikou *et al*., 2011;

Soumana *et al.*, 2013; Aksoy *et al.*, 2014; Griffith *et al.*, 2018; Kame-Ngasse *et al.*, 2018). Although the mechanism that underlies this correlation has never been experimentally validated, one theory posits that *Sodalis* residing in tsetse's midgut secretes chitinase that degrades the fly's peritrophic matrix (PM) (Welburn *et al.*, 1993; Rose *et al.*, 2014). This process releases *N*-acetyl-D-glucosamine that serves as a prominent carbon source for *Sodalis* and may facilitate trypanosome infection establishment by 'mopping up' trypanolytic lectins (Welburn and Maudlin, 1992; Welburn *et al.*, 1993, 1994). Additionally, *Sodalis*-mediated degradation of tsetse's PM may enhance the ability of trypanosomes to circumvent the barrier, which they must do in order to establish an infection in the ectoperitrophic space of the fly's midgut (Weiss *et al.*, 2013, 2014; Rose *et al.*, 2020). More research is required to determine if and how *Sodalis* impacts the establishment of trypanosome infections in tsetse.

Tsetse populations can also harbour two additional vertically transmitted parasitic endosymbionts, *Wolbachia* and *Spiroplasma*. Unlike *Wigglesworthia* and *Sodalis*, *Wolbachia* and *Spiroplasma* infect host gonadal tissues and are vertically transmitted to developing progeny during embryogenesis (O'Neill *et al.*, 1993; Cheng *et al.*, 2000; Doudoumis *et al.*, 2017). *Wolbachia* infections induce a variety of reproductive abnormalities in their hosts, the most common of which is called cytoplasmic incompatibility (CI). CI occurs when a *Wolbachia*-infected male mates with an uninfected female, or a female that houses a different *Wolbachia* strain or more than one strain (the latter situation is referred to as a superinfection) (Werren *et al.*, 2008; Shropshire *et al.*, 2020). Under these circumstances, sperm enters the egg but does not successfully transfer DNA, thus resulting in developmental arrest during embryogenesis (Zabalou *et al.*, 2004; Shropshire *et al.*, 2020). The ability of *Wolbachia* to induce CI in tsetse was investigated by mating symbiont-cured females with males that harboured their complete endogenous microbiome. These crosses resulted in the presentation of a robust CI

phenotype during embryogenesis of subsequent offspring (Alam *et al.*, 2011). Because CI confers a reproductive advantage to infected females over their uninfected counterparts (they can mate with males that do or do not house *Wolbachia*) and *Wolbachia*-infected females transmit the symbiont to their progeny, *Wolbachia* may be used as a driver to replace trypanosome-susceptible tsetse populations with flies that are being developed to present parasite-resistant phenotypes (Aksoy *et al.*, 2001). In addition, CI will enable the spread of all maternally transmitted agents, such as other maternally transmitted endosymbionts that can be used to engineer paratransgenic tsetse. Furthermore, *Wolbachia* induces parasite refractory phenotypes in several arthropod vectors (McGraw and O'Neill, 2013; Ferreira *et al.*, 2020) (though the bacterium's role in tsetse vector competence requires further investigation). This characteristic has led to applications in geographically distinct regions (e.g., USA, Australia, Brazil) where insects infected with *Wolbachia*, both naturally and experimentally, are released to replace their susceptible counterparts as a means of reducing disease (Hoffmann *et al.*, 2011; Caragata *et al.*, 2016; Joubert *et al.*, 2016; Flores and O'Neill, 2018).

Interestingly, *Wolbachia* and *Spiroplasma* infect tsetse species that belong to the *Morsitans* and *Palpalis* subgroups, respectively (Alam *et al.*, 2012; Doudoumis *et al.*, 2012). The infection prevalence and abundance of each bacterium vary within and between natural tsetse populations and laboratory-reared tsetse lines (Cheng *et al.*, 2000; Doudoumis *et al.*, 2017; Schneider *et al.*, 2019). The regulation of *Wolbachia* infection prevalence in natural populations has applied significance, although information is limited. Similarly, *Spiroplasma* effects on tsetse's physiology should be further investigated, as infections with the bacterium may also have translational implications. Similar to *Wolbachia*-induced pathogen resistance, laboratory-reared *Glossina fuscipes fuscipes* that house *Spiroplasma* are significantly more refractory to infection with trypanosomes than are individuals that lack the bacterium (Schneider *et al.*, 2019). Furthermore, because *G. f. fuscipes*

competes with *Spiroplasma* for reproductively critical circulating lipids, females that house the bacterium exhibit significantly decreased fecundity. Finally, sperm from *Spiroplasma*-infected male *G. f. fuscipes* present compromised motility, which would likely put them at a reproductive disadvantage compared with uninfected males (Son *et al.*, 2021). These *Spiroplasma*-induced reproductive phenotypes should be taken into consideration when optimizing *G. f. fuscipes* control strategies, such as SIT, that require the mass rearing of large quantities of flies.

13.3 Utilizing Endogenous Endosymbionts for Tsetse Paratransgenesis

Transgenesis has been used to express foreign genes ectopically in several important insect vectors, including a mosquito that transmits malaria in Asia (*Anopheles stephensi*) as well as the yellow fever mosquito, *Aedes aegypti* (Terenius *et al.*, 2008; Wang and Jacobs-Lorena, 2013) (see Bottino-Rojas and James, Chapter 11; Franz, Chapter 22, this volume). Under these circumstances genetic transformation of host insects is achieved by microinjecting a transposable element (plasmid or viral vectors) into syncytial embryos, followed by integration of the DNA into the chromosomes. This process is called germline transformation (see O'Brochta, Chapter 1, this volume). Tsetse's viviparous reproductive biology prohibits the use of germline transformation as a means of genetically modifying this insect. Thus, an alternative approach called paratransgenesis has been developed. Paratransgenesis is a process that involves genetically modifying a vector's endosymbiotic bacteria so that they express genes that encode anti-pathogen effector molecules, and/or molecules that modify vector physiology so as to make the host environment less hospitable for pathogens, which then can be rapidly spread among vector populations. In tsetse this strategy relies on the ability to cultivate and genetically modify tsetse's commensal symbiont *Sodalis* so that the bacterium expresses and releases

an effector molecule that interferes with parasite viability (Beard *et al.*, 1993a,b; Kariithi *et al.*, 2018). Tsetse are then recolonized with the modified *Sodalis*, which confers parasite resistance traits (Fig. 13.1).

13.3.1 Recombinant *Sodalis* is well suited for tsetse paratransgenesis

Sodalis is well suited to express foreign anti-trypanosomal products in tsetse flies for several reasons. First, *Sodalis* resides extracellularly in tsetse's gut near pathogenic trypanosomes. Thus, trypanocidal substances released by genetically modified bacterial cells are more likely to interfere with parasite transmissions. Secondly, *Sodalis* can be cultured (both in liquid media and on agar plates) (Dale and Maudlin, 1999; Hall *et al.*, 2020) and genetically modified *in vitro* to express foreign genes of interest (Beard *et al.*, 1993a; Pontes and Dale, 2006; Kendra *et al.*, 2020; Keller *et al.*, 2021). Along this line, *Sodalis* is highly resistant to several tsetse immune molecules, including the antimicrobial peptide attacin (Hu and Aksoy, 2005) and peptidoglycan recognition protein-LB (Wang and Aksoy, 2012). Both of these molecules, which are naturally expressed to limit parasite infection in this fly, may be good candidates for paratransgenic expression by *Sodalis* (Hu and Aksoy, 2005; Wang *et al.*, 2009; Wang and Aksoy, 2012). Genetically modified *Sodalis* can then be reintroduced into tsetse via one or a combination of routes: thoracic microinjection into adult flies (Aksoy *et al.*, 2008), *per os* in a bloodmeal (Maltz *et al.*, 2012), and/or microinjection into 3rd-instar larva (De Vooght *et al.*, 2018). While a previous study indicated that the fitness of the transformed *Sodalis* and paratransgenic tsetse is not compromised in comparison with their wild-type counterparts (Weiss *et al.*, 2006), this outcome in different environments is likely dependent on several ecological and genetic factors. An important requirement of this method is that the transformed *Sodalis* are passed on to future tsetse progeny through either maternal or paternal vertical transmission. Current
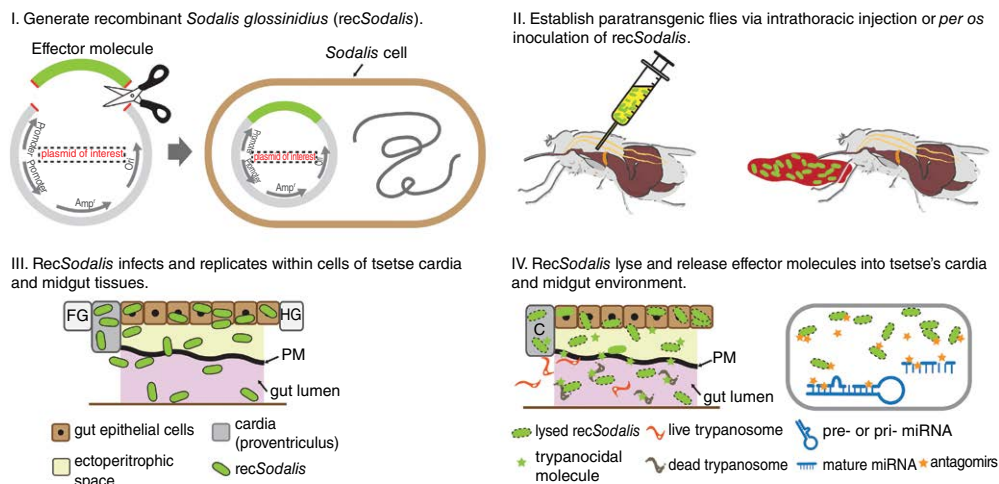
Fig. 13.1. Schematic representation of tsetse paratransgenesis. (I) To generate recombinant *Sodalis glossinidius* (rec*Sodalis*), a plasmid construct is engineered to express a gene that encodes an effector molecule. Recombinant effector molecules such as antimicrobial peptides (e.g., attacin, BMAP-27, PGRP-LB) and single-chain antibodies and nanobodies can have a direct inhibitory effect on trypanosomes, while double-stranded RNAs and microRNA antagomirs may alter the physiological homeostasis of the midgut environment in a way that indirectly impacts trypanosome transmission through the fly. The construct is then transferred (using one of a number of processes, including but not limited to electroporation, conjugation and/or transduction) into cultured wild-type *Sodalis* cells where it is maintained extrachromosomally. **(II)** Paratransgenic tsetse are generated by injecting rec*Sodalis* into the fly thorax or inoculating the bacteria into a heat-inactivated blood meal. **(III)** rec*Sodalis* infects and replicates within cells of tsetse's cardia and midgut tissues. FG, foregut; HG, hindgut; PM, peritrophic matrix. **(IV)** Intracellular rec *Sodalis* releases effector molecules into tsetse's cardia and midgut environments via active secretion (when the exogenous DNA also encodes a secretion signal) and/or bacterial cell lysis. Released effector molecules then directly or indirectly interfere with parasite transmission through the fly.

results demonstrate low-frequency transmission to progeny, which is an outcome that must be optimized (De Vooght *et al.*, 2015; Medina Munoz *et al.*, 2020). Additionally, *Sodalis* isolates from one tsetse species can be transinfected into different tsetse species to streamline the paratransgenesis process (Weiss *et al.*, 2006). Lastly, the *Sodalis* genome is completely sequenced and annotated and this information will serve as a valuable resource that can be exploited to improve the efficiency of our expression system (Toh *et al.*, 2006).

13.3.2 Identification and expression of anti-trypanosomal effector molecules

The importance of identifying trypanocidal molecules has intensified with the availability of a tsetse paratransgenesis system. Two types of effector molecules may be expressed to kill trypanosomes: (i) transmission-blocking agents; and (ii) host effector antimicrobial peptides.

Transmission-blocking agents

Transmission-blocking agents, such as monoclonal antibodies, can disrupt parasite development and pathogenicity by binding parasite proteins necessary for these events to occur. Several molecules that target the major surface protein of insect-stage parasites (procyclic *T. brucei*) have been reported (Nantulya and Moloo, 1988). For practical purposes, these molecules can be expressed as target-specific single-chain antibody fragments from one gene (Sc-Fv). A molecule of this nature was successfully expressed by

symbiotic bacteria (*Rhodococcus rhodnii*) living in the gut of the triatome bug, *Rhodnius prolixus* (insect vector of New World trypanosomes) (Durvasula *et al.*, 1999) (see Hurwitz *et al.*, Chapter 14, this volume). In this experiment, functional single-chain antibody rDB3 (which encodes murine V(H)/K that binds progesterone) was exported into the host insect's gut lumen. However, Sc-Fvs are large and architecturally complex (Whitlow *et al.*, 1993), which may limit their practical application as paratransgenic effectors. Recently, small antigen-binding fragments, called nanobodies (Nbs), were shown to target specific trypanosome variant-specific surface glycoprotein (VSG) domains (De Vooght *et al.*, 2012, 2018). These molecules exhibit *in vitro* and *in vivo* toxicity against *T. brucei* parasites by inhibiting vital trypanosome physiological processes (Caljon *et al.*, 2012). As further proof of their effectiveness as paratransgenic effector molecules, a nanobody (Nb_An33) was expressed in *Sodalis* that specifically recognizes a conserved VSG epitope that is otherwise inaccessible to larger conventional antibodies (Stijlemans *et al.*, 2004). Furthermore, inclusion of the pectate lysateB (pelB) signal peptide into the Nb_An33 expression construct resulted in the export of recombinant nanobody to the bacterial periplasm (De Vooght *et al.*, 2012). These findings indicate that nanobodies may serve as efficient effector molecules in paratransgenic expression systems.

Antimicrobial peptides

Small antimicrobial peptides are one component of the innate immune system of many higher multicellular organisms. These well-studied substances are produced by tsetse's immune tissues when challenged by a pathogen (or pathogens) and they may be also responsible for the pathogen-refractory phenotypes of many vector species. Three such molecules have been identified in tsetse: attacin, cecropin and daptericin (Hao *et al.*, 2001; Pais *et al.*, 2008). These three peptides are synthesized through the activation of the immune deficiency (Imd) signalling pathway (Hu and Aksoy, 2006). Induction of the Imd pathway limits the establishment

of trypanosomes in tsetse's midgut. Furthermore, one of these peptides, attacin, exhibits anti-trypanosomal activity *in vitro* (Hu and Aksoy, 2005). Antimicrobial peptide gene(s) from other animals, or synthetic peptides, may also be suitable for production via paratransgenic expression. One such antimicrobial peptide, BMAP-27, is produced by bovine neutrophils. Experiments by Haines *et al.* (2003) revealed that BMAP-27 is highly lethal to both bloodstream-form and procyclic-form trypanosomes. Further experiments revealed that *Sodalis* is resistant to 65-fold higher concentrations of BMAP-27 than bloodstream-form trypanosomes. These results indicate that expression of this peptide by *Sodalis* may be possible and that it may be very useful in paratransgenesis experiments as an anti-trypanosomal effector molecule.

Another promising candidate for use in the tsetse paratransgenic system is the human trypanocide apolipoprotein L-1 (apoL-1). This protein, which is a component of normal human serum, lyses trypanosomes that do not cause HAT (Vanhamme and Pays, 2004). Human lysis-resistant trypanosomes express a surface protein called serum resistance associated (SRA) protein, which inactivates apoL-1 (by interacting with the protein's C-terminus, resulting in parasite survival and proliferation (Xong *et al.*, 1998)). However, *T. b. rhodesiense* is susceptible when incubated with a truncated version of apoL-1 (Tr-apoL-1) that lacks a C-terminal SRA-interacting domain (Vanhamme and Pays, 2004). As a means of developing this protein for potential HAT therapy, Baral *et al.* (2006) fused Tr-apoL-1 to the NbAn33 single-domain nanobody, thus allowing the conjugate to out-compete endogenous apoL-1. Treatment of trypanosome-infected mice with NbAn33–Tr-apoL-1 caused no adverse physiological effects and definitively cured animals of parasites. This type of nanobody-conjugated trypanocide, combined with a *Sodalis*-specific secretion signal, has promising potential to increase tsetse's refractoriness to infection.

An additional trypanocidal effector that could be useful in paratransgenesis is the tsetse protein PGRP-LB. This molecule is

expressed in the epithelial tissues in response to the presence of microbial PGN (Wang *et al.*, 2009). PGRP-LB is a secreted host protein that also exhibits antiparasitic activity *in vitro*. Although recPGRPL-LB has antimicrobial activity against *E. coli*, *Sodalis* is resistant to its activity at similar concentrations (Wang and Aksoy, 2012). Thus, paratransgenic expression of PGRP-LB in tsetse's gut could render the environment inhospitable to incoming parasites.

13.3.3 Paratransgenic manipulation of tsetse midgut physiology to alter parasite infection dynamics

Small molecules that contribute to the regulatory pathways during parasite transmissions can be used as a target of paratransgenic approach. For instance, small RNAseq revealed that a small group of microRNAs (miRNAs) differentially expressed in tsetse's gut upon trypanosome exposure; when one particular miRNA, *miR275*, was knocked down using synthetic antagomir, the integrity of peritrophic matrix (PM) was compromised (Aksoy *et al.*, 2016). PM acts as a physical barrier that lines tsetse's gut and protects tsetse from food abrasion and pathogenic microorganisms (Hegedus *et al.*, 2009; Aksoy, 2019). Thus, regulating the expression of tsetse *miR275* (and potentially other miRNAs) can be useful in manipulating parasite infection outcomes. Yang *et al.* (2021) engineered two strains of recombinant *Sodalis* (rec*Sodalis*), one of which expresses tandem repeats of antagomir-275 to knock down *miR275* and a control strain that expresses a scrambled *miR275* sequence. These two strains of rec*Sodalis* were then reintroduced into teneral tsetse flies *per os* in a bloodmeal to generate two paratransgenic fly lines designated as $Gmm^{3xant-miR275}$ and $Gmm^{Scr-275}$, respectively. Rec*Sodalis* was detected in multiple tsetse tissues, including cardia and midgut, and the *miR275* levels were significantly reduced in the midgut of $Gmm^{3xant-miR275}$ flies. Several robust phenotypes associated with compromised PM structure were observed in $Gmm^{3xant-miR275}$ compared with $Gmm^{Scr-275}$ flies. For example, guts from $Gmm^{3xant-miR275}$ flies weighed significantly more than those from $Gmm^{Scr-275}$ individuals, which indicates that the reduction in *miR275* expression obstructed blood digestion or renal excretion of the flies. Additionally, $Gmm^{3xant-miR275}$ flies were significantly more susceptible to infection with trypanosomes than were control flies. These phenotypes exhibited by $Gmm^{3xant-miR275}$ flies mirror their midgut transcriptomic profiles, which present the differential expression (in comparison with the same tissue in $Gmm^{Scr-275}$ individuals) of genes that encode PM-associated proteins, digestive enzymes, and secreted products found previously in tsetse saliva (Zhao *et al.*, 2015). These findings represent proof-of-concept that paratransgenic expression of microRNA antagomirs can be used to directly manipulate parasite infection outcomes or serve as a platform for studying microRNA regulatory pathways and mechanism in tsetse flies as well as other arthropod vector model systems.

13.4 Utilizing Exogenous Bacteria for Tsetse Paratransgenesis

Bacterial taxa not found naturally in tsetse's gut can be experimentally introduced into the environment for the purposes of altering the fly's vector competence. One such bacterium is *Sodalis praecaptivus*, which is the free-living ancestral strain of *Sodalis* spp. that currently resides endosymbiotically within numerous insect taxa, including tsetse (Clayton *et al.*, 2012; Chari *et al.*, 2015). *S. praecaptivus* can be cultured and genetically modified (Enomoto *et al.*, 2017). Additionally, when microinjected into the thorax of *G. morsitans*, the bacterium persists within the fly for at least 50 days and has no adverse effects on its host's physiological well-being. Finally, microinjected *S. praecaptivus* successfully colonized reproductive tissues of both male and female tsetse, and is vertically transmitted from parents to offspring (interestingly, male flies had to be infected with the bacterium in order for vertical transmission to

occur) (Medina Munoz *et al.*, 2020). Taken together, these characteristics make *S. praecaptivus* a promising candidate for the paratransgenic expression of anti-trypanosomal effector molecules within tsetse.

Kosakonia cowanii Zambiae (*Kco_Z*) represents another exogenous bacterium that exhibits potential as a candidate for tsetse paratransgenesis. *Kco_Z*, which was originally isolated from field-captured *Anopheles gambiae* mosquitoes, induces a *Plasmodium* refractory phenotype in laboratory-reared mosquitoes by producing reactive oxygen intermediates (ROIs) that are directly toxic to the parasites (Cirimotich *et al.*, 2011; Dennison *et al.*, 2016). When inoculated into tsetse via a bloodmeal, *Kco_Z* stably colonizes the fly's gut for at least 28 days and does not impact host survival. Tsetse that house *Kco_Z* are unusually resistant to infection with African trypanosomes. However, unlike in *An. gambiae*, the *Kco_Z*-induced trypanocidal phenotype in tsetse does not result from bacterial production of ROIs. Instead, *Kco_Z* acidifies tsetse's midgut, which creates an environment that is hostile to trypanosomes (Weiss *et al.*, 2019).

Paratransgenic tsetse colonized with *Kco_Z* may be particularly well suited to enhance the efficacy of SIT. Tsetse SIT involves the experimental saturation of a target environment with irradiated, sterilized males that outcompete native males for female mates who subsequently fail to produce viable offspring. This outcome is very effective at reducing fly populations and thus disease transmission (Abd-Alla *et al.*, 2013; Vreysen *et al.*, 2014). However, one shortcoming of tsetse SIT is that released males feed on local vertebrate trypanosome reservoirs and are thus all potential disease vectors. This impediment may be overcome by releasing males that harbour *Kco_Z* in their midgut. These males, which are equally fit in comparison with their wild-type counterparts (Weiss *et al.*, 2019), would present *Kco_Z*-mediated refractoriness to trypanosome infection and thus present a significantly reduced public health threat. This effect could be further enhanced by colonizing tsetse with genetically modified *Kco_Z* that produce trypanocidal effector molecules.

13.5 Mechanisms to Drive Parasite-Resistant Tsetse Phenotypes into Natural Populations

13.5.1 Exploiting *Wolbachia*-mediated mating incompatibilities

The ability to spread laboratory-generated, parasite-resistant phenotypes into natural field populations is a crucial component of using transgenic and paratransgenic methods to control the spread of vector-borne diseases. One such mechanism results from reproductive abnormalities mediated by *Wolbachia* infections. *Wolbachia*-mediated spread of infected insects occurs in natural populations of *Drosophila* (Turelli and Hoffmann, 1995, 1999) as well as *Ae. aegypti* (Hoffmann *et al.*, 2011). The presence of *Wolbachia* infections in laboratory lines of tsetse, as well as in natural populations, has been described. The *Wolbachia* infection prevalence in *Glossina austeni* and *G. brevipalpis* populations analysed from Kenya in the mid-1990s was reported to be 48% and 98% (Cheng *et al.*, 2000). A recent analysis of *G. austeni* from the same populations in Kenya found 100% infection prevalence in this species, suggesting that *Wolbachia* has reached fixation in this population (Wamwiri *et al.*, 2013). Heterogeneous *Wolbachia* infections have also been noted in different tsetse field populations (Alam *et al.*, 2012; Doudoumis *et al.*, 2012, 2013; Symula *et al.*, 2013; Kante *et al.*, 2018; Simo *et al.*, 2019). Additionally, *Wolbachia*-free *G. m. morsitans* can be generated by treating fertile females with tetracycline in the presence of yeast extract-supplemented bloodmeals (Alam *et al.*, 2011). This treatment eliminates *Wolbachia* and maintains maternal fecundity so that these females give rise to aposymbiotic progeny. Crosses between wild-type males and *Wolbachia*-cured females show evidence of strong CI expression in tsetse. *Wolbachia*-induced CI may be exploitable to improve the efficacy of the existing control tools (such as SIT) and used in new methods that aim to replace susceptible populations with their counterparts modified to present parasite-resistant phenotypes. In the context

of SIT programmes, utilization of *Wolbachia*-infected males for SIT releases would result in incompatibility with uninfected field populations. Hence, it may be possible to lower the dose of irradiation to which the flies are subjected prior to release, thus achieving greater fitness for SIT males. If the target population is already infected with a *Wolbachia*, then releasing males that carry a different strain of this bacterium would ensure incompatibility through the activation of bidirectional CI (CI that occurs following matings between individuals that harbour different *Wolbachia* strains).

Because all endosymbiotic bacteria are maternally transmitted into developing tsetse larva, released paratransgenic flies that house *Wolbachia* and recombinant *Sodalis* that express effector proteins would likely move through and outcompete natural, wild-type populations. This strategy would require that transmission of *Sodalis* from mother to progeny, and the coupling of *Sodalis* and *Wolbachia*, remains high. While laboratory experiments demonstrate perfect transmission (Alam *et al.*, 2011), field data remain to be obtained for these parameters. The availability of parasite-resistant lines would allow for the development of alternative control strategies that target replacement of disease-susceptible populations with their resistant counterparts. In this case, CI can similarly be harnessed to drive the modified phenotypes in nature by releasing both male and female adults that carry an incompatible *Wolbachia* strain.

13.5.2   Modelling the efficacy of paratransgenic control

When *Wolbachia* is present at high frequency in a population, females infected with this bacterium have a higher reproductive success than their non-infected counterparts, assuming CI is expressed. Furthermore, assuming perfect transmission from the mother to all of her progeny, infected females give rise to *Wolbachia*-infected individuals. At low frequency, *Wolbachia*-infected females may have a fitness disadvantage if *Wolbachia* reduces

egg count or increases mortality. Thus, a threshold in *Wolbachia* frequency may exist above which *Wolbachia* increases tsetse fitness and is therefore driven to fixation (Turelli and Hoffmann, 1999; Dobson, 2003). Continuous-time modelling of *Wolbachia*–tsetse dynamics, based on known reproductive rates of tsetse mating pairs, suggested that the time required to reach fixation following the release of *Wolbachia*-infected tsetse (assuming a release population that equals 10% of the native population) could be relatively short (median value of 529 days) (Alam *et al.*, 2011).

13.5.3   Polyandry and cytoplasmic incompatibility

Genetic data provide insights into the reproductive biology of tsetse, particularly on the number of times a female can mate and maintain sperm from different mates in the wild (polyandry). Polyandry may constitute a critical factor in cases of reinfestation of cleared areas or of residual populations. Additionally, polyandry may enhance the reproductive potential of re-invading propagules in terms of their effective population size. Remating can also adversely impact the success of genetic control methods, such as SIT or incompatible insect technique (IIT), or the success of approaches that aim to replace disease-susceptible natural populations with modified resistant phenotypes. These outcomes could occur if sperm contributed from fertile and sterile males, or from uninfected and *Wolbachia*-infected males, is used differentially. Our analysis of two tsetse populations indicated that remating is common (57% in Kabukanga in western Uganda and 33% in Buvuma Island in Lake Victoria) (Bonomi *et al.*, 2011). Population age structure may influence remating frequency. Considering the seasonal demographic changes that tsetse undergoes during the dry and wet seasons, control programmes based on SIT should release large numbers of sterile males in the dry season, even in residual surviving target populations. The role of polyandry and the

failure to produce offspring due to incompatible first matings may affect the likelihood of re-matings among female tsetse. This could be particularly relevant in areas where flies carry heterogeneous *Wolbachia* infections and where mating incompatibilities mediated by CI could fuel re-matings. Given the high prevalence of polyandry, sperm use in polyandrous females remains to be determined. The efficacy of matings with sterile males can be reduced if there is preferential use of wild-type (WT) sperm in the context of SIT programmes. Similarly, the efficacy of CI-based replacement or IIT methods would be compromised if there were preferential use of WT sperm. Mathematical models with empirical data can provide insights into how polyandry and sperm use affect the success of paratransgenic control methods.

13.6 Conclusions

Effective management of insect disease vectors and the pathogens they transmit will require a coordinated effort that combines the use of several different suppression strategies. This chapter provides an overview of one such strategy, called paratransgenesis, and how it can be used to control the spread of African trypanosomes by the tsetse fly. Recent technological breakthroughs, including advances in high-throughput sequencing methodologies and functional genomics, will allow us to learn more about the interactions between tsetse flies and their associated microorganisms. This knowledge can then be applied to increase the efficacy of the system. Additionally, more studies are necessary to determine how tsetse's natural microbiota might impact paratransgenesis and parasite transmission dynamics in the field. Finally, for disease control strategies that employ genetically modified organisms to succeed, they must be accepted by societies in which they will be implemented. Thus, as policies are being developed and adopted by disease-endemic countries, data on safe and effective use of these methods (including containment of modified symbionts and expressed transgenes) must be obtained. While paratransgenesis provides a powerful tool to investigate the fundamental aspects of tsetse physiology and biology, its ultimate application as a disease control method will require input from scientists and governments in these countries.

References

Abd-Alla, A.M., Bergoin, M., Parker, A.G., Maniania, N.K., Vlak, J.M. *et al.* (2013) Improving Sterile Insect Technique (SIT) for tsetse flies through research on their symbionts and pathogens. *Journal of Invertebrate Pathology* 112 (Suppl.) S2–10.

Akman, L., Yamashita, A., Watanabe, H., Oshima, K., Shiba, T. *et al.* (2002) Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. *Nature Genetics* 32, 402–407.

Aksoy, E., Telleria, E.L., Echodu, R., Wu, Y., Okedi, L.M. *et al.* (2014) Analysis of multiple tsetse fly populations in Uganda reveals limited diversity and species-specific gut microbiota. *Applied and Environmental Microbiology* 80, 4301–4312.

Aksoy, E., Vigneron, A., Bing, X., Zhao, X., O'Neill, M. *et al.* (2016) Mammalian African trypanosome VSG coat enhances tsetse's vector competence. *Proceedings of the National Academy of Sciences USA* 113, 6961–6966.

Aksoy, S. (1995) *Wigglesworthia* gen. nov. and *Wigglesworthia glossinidia* sp. nov., taxa consisting of the mycetocyte-associated, primary endosymbionts of tsetse flies. *International Journal of Systematic Bacteriology* 45, 848–851.

Aksoy, S. (2019) Tsetse peritrophic matrix influences for trypanosome transmission. *Journal of Insect Physiology* 118, 103919.

Aksoy, S. (2000) Tsetse – a haven for microorganisms. *Parasitology Today* 16, 114–118.

Aksoy, S., Chen, X. and Hypsa, V. (1997) Phylogeny and potential transmission routes of midgut-associated endosymbionts of tsetse (Diptera: Glossinidae). *Insect Molecular Biology* 6, 183–190.

Aksoy, S., Maudlin, I., Dale, C., Robinson, A.S. and O'Neill, S.L. (2001) Prospects for control of African tryp-
anosomiasis by tsetse vector manipulation. *Trends in Parasitology* 17, 29–35.

Aksoy, S., Weiss, B. and Attardo, G. (2008) Paratransgenesis applied for control of tsetse transmitted sleep-
ing sickness. *Advances in Experimental Medicine and Biology* 627, 35–48.

Aksoy, S., Buscher, P., Lehane, M., Solano, P. and Van Den Abbeele, J. (2017) Human African trypanosom-
iasis control: achievements and challenges. *PLoS Neglected Tropical Diseases* 11, e0005454.

Alam, U., Medlock, J., Brelsfoard, C., Pais, R., Lohs, C. *et al.* (2011) *Wolbachia* symbiont infections induce
strong cytoplasmic incompatibility in the tsetse fly *Glossina morsitans*. *PLoS Pathogens* 7, e1002415.

Alam, U., Hyseni, C., Symula, R.E., Brelsfoard, C., Wu, Y. *et al.* (2012) Implications of microfauna–host inter-
actions for trypanosome transmission dynamics in *Glossina fuscipes fuscipes* in Uganda. *Applied
and Environmental Microbiology* 78, 4627–4637.

Attardo, G.M., Lohs, C., Heddi, A., Alam, U.H., Yildirim, S. and Aksoy, S. (2008) Analysis of milk gland struc-
ture and function in *Glossina morsitans*: milk protein production, symbiont populations and fecundity.
Journal of Insect Physiology 54, 1236–1242.

Attardo, G.M., Tam, N., Parkinson, D., Mack, L.K., Zahnle, X.J. *et al.* (2020) Interpreting morphological
adaptations associated with viviparity in the tsetse fly *Glossina morsitans* (Westwood) by three-
dimensional analysis. *Insects* 11, 651.

Balmand, S., Lohs, C., Aksoy, S. and Heddi, A. (2013) Tissue distribution and transmission routes for the
tsetse fly endosymbionts. *Journal of Invertebrate Pathology* 112 (Suppl.), S116–122.

Baral, T.N., Magez, S., Stijlemans, B., Conrath, K., Vanhollebeke, B. *et al.* (2006) Experimental therapy of
African trypanosomiasis with a nanobody-conjugated human trypanolytic factor. *Nature Medicine* 12,
580–584.

Barrett, M.P. (2018) The elimination of human African trypanosomiasis is in sight: Report from the third
WHO stakeholders meeting on elimination of *gambiense* human African trypanosomiasis. *PLoS Neg-
lected Tropical Diseases* 12, e0006925.

Beard, C.B., O'Neill, S.L., Mason, P., Mandelco, L., Woese, C.R. *et al.* (1993a) Genetic transformation and
phylogeny of bacterial symbionts from tsetse. *Insect Molecular Biology* 1, 123–131.

Beard, C.B., O'Neill, S.L., Tesh, R.B., Richards, F.F. and Aksoy, S. (1993b) Modification of arthropod vector
competence via symbiotic bacteria. *Parasitology Today* 9, 179–183.

Benoit, J.B., Attardo, G.M., Baumann, A.A., Michalkova, V. and Aksoy, S. (2015) Adenotrophic viviparity
in tsetse flies: potential for population control and as an insect model for lactation. *Annual Review of
Entomology* 60, 351–371.

Benoit, J.B., Vigneron, A., Broderick, N.A., Wu, Y., Sun, J.S. *et al.* (2017) Symbiont-induced odorant binding
proteins mediate insect host hematopoiesis. *Elife* 6, e19535.

Bonomi, A., Bassetti, F., Gabrieli, P., Beadell, J., Falchetto, M. *et al.* (2011) Polyandry is a common event in
wild populations of the tsetse fly *Glossina fuscipes fuscipes* and may impact population reduction
measures. *PLoS Neglected Tropical Diseases* 5, e1190.

Caljon, G., Stijlemans, B., Saerens, D., Van Den Abbeele, J., Muyldermans, S. *et al.* (2012) Affinity is an
important determinant of the anti-trypanosome activity of nanobodies. *PLoS Neglected Tropical Dis-
eases* 6, e1902.

Caragata, E.P., Dutra, H.L.C. and Moreira, L.A. (2016) Exploiting intimate relationships: controlling mosquito-
transmitted disease with *Wolbachia*. *Trends in Parasitology* 32, 207–218.

Chari, A., Oakeson, K.F., Enomoto, S., Jackson, D.G., Fisher, M.A. and Dale, C. (2015) Phenotypic charac-
terization of *Sodalis praecaptivus* sp. nov., a close non-insect-associated member of the *Sodalis*-
allied lineage of insect endosymbionts. *International Journal of Systematic and Evolutionary Microbiology*
65, 1400–1405.

Chen, X., Li, S. and Aksoy, S. (1999) Concordant evolution of a symbiont with its host insect species: mo-
lecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont, *Wigglesworthia
glossinidia*. *Journal of Molecular Evolution* 48, 49–58.

Cheng, Q. and Aksoy, S. (1999) Tissue tropism, transmission and expression of foreign genes *in vivo* in
midgut symbionts of tsetse flies. *Insect Molecular Biology* 8, 125–132.

Cheng, Q., Ruel, T.D., Zhou, W., Moloo, S.K., Majiwa, P. *et al.* (2000) Tissue distribution and prevalence of
Wolbachia infections in tsetse flies, *Glossina* spp. *Medical and Veterinary Entomology* 14, 44–50.

Cirimotich, C.M., Dong, Y., Clayton, A.M., Sandiford, S.L., Souza-Neto, J.A. *et al.* (2011) Natural
microbe-mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*. *Science* 332,
855–858.

Clayton, A.L., Oakeson, K.F., Gutin, M., Pontes, A., Dunn, D.M. *et al.* (2012) A novel human-infection-derived bacterium provides insights into the evolutionary origins of mutualistic insect-bacterial symbioses. *PLoS Genetics* 8, e1002990.

Dale, C. and Maudlin, I. (1999) *Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans morsitans*. *International Journal of Systematic Bacteriology* 49 (Pt 1), 267-275.

De Vooght, L., Caljon, G., Stijlemans, B., De Baetselier, P., Coosemans, M. and Van den Abbeele, J. (2012) Expression and extracellular release of a functional anti-trypanosome Nanobody(R) in *Sodalis glossinidius*, a bacterial symbiont of the tsetse fly. *Microbial Cell Factories* 11, 23.

De Vooght, L., Caljon, G., De Ridder, K. and Van Den Abbeele, J. (2014) Delivery of a functional anti-trypanosome nanobody in different tsetse fly tissues via a bacterial symbiont, *Sodalis glossinidius. Microbial Cell Factories* 13, 156.

De Vooght, L., Caljon, G., Van Hees, J. and Van Den Abbeele, J. (2015) Paternal transmission of a secondary symbiont during mating in the viviparous tsetse fly. *Molecular Biology and Evolution* 32, 1977–1980.

De Vooght, L., Van Keer, S. and Van Den Abbeele, J. (2018) Towards improving tsetse fly paratransgenesis: stable colonization of *Glossina morsitans morsitans* with genetically modified *Sodalis*. *BMC Microbiology* 18, 165.

Dennison, N.J., Saraiva, R.G., Cirimotich, C.M., Mlambo, G., Mongodin, E.F. and Dimopoulos, G. (2016) Functional genomic analyses of *Enterobacter*, *Anopheles* and *Plasmodium* reciprocal interactions that impact vector competence. *Malaria Journal* 15, 425.

Diall, O., Cecchi, G., Wanda, G., Argiles-Herrero, R., Vreysen, M.J.B. *et al.* (2017) Developing a progressive control pathway for African animal trypanosomosis. *Trends in Parasitology* 33, 499–509.

Dobson, S.L. (2003) Reversing *Wolbachia*-based population replacement. *Trends in Parasitology* 19, 128–133.

Doudoumis, V., Tsiamis, G., Wamwiri, F., Brelsfoard, C., Alam, U. *et al.* (2012) Detection and characterization of *Wolbachia* infections in laboratory and natural populations of different species of tsetse flies (genus *Glossina*). *BMC Microbiology* 12 (Suppl. 1), S3.

Doudoumis, V., Alam, U., Aksoy, E., Abd-Alla, A.M., Tsiamis, G. *et al.* (2013) Tsetse–*Wolbachia* symbiosis comes of age and has great potential for pest and disease control. *Journal of Invertebrate Pathology* 112 (Suppl.), S94–103.

Doudoumis, V., Blow, F., Saridaki, A., Augustinos, A., Dyer, N.A. *et al.* (2017) Challenging the *Wigglesworthia*, *Sodalis*, *Wolbachia* symbiosis dogma in tsetse flies: *Spiroplasma* is present in both laboratory and natural populations. *Scientific Reports* 7, 4699.

Durvasula, R.V., Gumbs, A., Panackal, A., Kruglov, O., Taneja, J. *et al.* (1999) Expression of a functional antibody fragment in the gut of *Rhodnius prolixus* via transgenic bacterial symbiont *Rhodococcus rhodnii*. *Medical and Veterinary Entomology* 13, 115–119.

Enomoto, S., Chari, A., Clayton, A.L. and Dale, C. (2017) Quorum sensing attenuates virulence in *Sodalis praecaptivus*. *Cell Host & Microbe* 21, 629–636, e625.

Erlandson, M.A., Toprak, U. and Hegedus, D.D. (2019) Role of the peritrophic matrix in insect–pathogen interactions. *Journal of Insect Physiology* 117, 103894.

Esterhuizen, J., Rayaisse, J.B., Tirados, I., Mpiana, S., Solano, P. *et al.* (2011) Improving the cost-effectiveness of visual devices for the control of riverine tsetse flies, the major vectors of human African trypanosomiasis. *PLoS Neglected Tropical Diseases* 5, e1257.

Farikou, O., Thevenon, S., Njiokou, F., Allal, F., Cuny, G. and Geiger, A. (2011) Genetic diversity and population structure of the secondary symbiont of tsetse flies, *Sodalis glossinidius*, in sleeping sickness foci in Cameroon. *PLoS Neglected Tropical Diseases* 5, e1281.

Ferreira, A.G., Fairlie, S. and Moreira, L.A. (2020) Insect vectors endosymbionts as solutions against diseases. *Current Opinion in Insect Science* 40, 56–61.

Flores, H.A. and O'Neill, S.L. (2018) Controlling vector-borne diseases by releasing modified mosquitoes. *Nature Reviews Microbiology* 16, 508–518.

Franco, J.R., Cecchi, G., Priotto, G., Paone, M., Diarra, A. *et al.* (2020) Monitoring the elimination of human African trypanosomiasis at continental and country level: Update to 2018. *PLoS Neglected Tropical Diseases* 14, e0008261.

Griffith, B.C., Weiss, B.L., Aksoy, E., Mireji, P.O., Auma, J.E. *et al.* (2018) Analysis of the gut-specific microbiome from field-captured tsetse flies, and its potential relevance to host trypanosome vector competence. *BMC Microbiology* 18, 146.

Haines, L.R., Hancock, R.E. and Pearson, T.W. (2003) Cationic antimicrobial peptide killing of African tryp-anosomes and *Sodalis glossinidius*, a bacterial symbiont of the insect vector of sleeping sickness. *Vector Borne and Zoonotic Disease* 3, 175–186.

Hall, R.J., Thorpe, S., Thomas, G.H. and Wood, A.J. (2020) Simulating the evolutionary trajectories of meta-bolic pathways for insect symbionts in the genus *Sodalis*. *Microbial Genomics* 6, mgen000378.

Hao, Z., Kasumba, I., Lehane, M.J., Gibson, W.C., Kwon, J. and Aksoy, S. (2001) Tsetse immune responses and trypanosome transmission: implications for the development of tsetse-based strategies to reduce trypanosomiasis. *Proceedings of the National Academy of Sciences USA* 98, 12648–12653.

Hegedus, D., Erlandson, M., Gillott, C. and Toprak, U. (2009) New insights into peritrophic matrix synthesis, architecture, and function. *Annual Review of Entomology* 54, 285–302.

Hoffmann, A.A., Montgomery, B.L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P.H. *et al.* (2011) Successful estab-lishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature* 476, 454–457.

Hu, Y. and Aksoy, S. (2005) An antimicrobial peptide with trypanocidal activity characterized from *Glossina morsitans morsitans*. *Insect Biochemistry and Molecular Biology* 35, 105–115.

Hu, C. and Aksoy, S. (2006) Innate immune responses regulate trypanosome parasite infection of the tsetse fly *Glossina morsitans morsitans*. *Molecular Microbiology* 60,1194–1204.

Joubert, D.A., Walker, T., Carrington, L.B., De Bruyne, J.T., Kien, D.H. *et al.* (2016) Establishment of a *Wol-bachia* superinfection in *Aedes aegypti* mosquitoes as a potential approach for future resistance management. *PLoS Pathogen* 12, e1005434.

Kame-Ngasse, G.I., Njiokou, F., Melachio-Tanekou, T.T., Farikou, O., Simo, G. and Geiger, A. (2018) Preva-lence of symbionts and trypanosome infections in tsetse flies of two villages of the 'Faro and Deo' division of the Adamawa region of Cameroon. *BMC Microbiology* 18, 159.

Kante, S.T., Melachio, T., Ofon, E., Njiokou, F. and Simo, G. (2018) Detection of *Wolbachia* and different trypanosome species in *Glossina palpalis palpalis* populations from three sleeping sickness foci of southern Cameroon. *Parasites and Vectors* 11, 630.

Kariithi, H.M., Meki, I.K., Schneider, D.I., De Vooght, L., Khamis, F.M. *et al.* (2018) Enhancing vector refrac-toriness to trypanosome infection: achievements, challenges and perspectives. *BMC Microbiology* 18, 179.

Keller, C.M., Kendra, C.G., Bruna, R.E., Craft, D. and Pontes, M.H. (2021) *Genetic modification of Sodalis species by DNA transduction.* mSphere 6, e01331-20.

Kendra, C.G., Keller, C.M., Bruna, R.E. and Pontes, M.H. (2020) *Conjugal DNA transfer in Sodalis glossin-idius, a maternally inherited symbiont of tsetse flies.* mSphere 5, e00864-20.

Lehane, M., Alfaroukh, I., Bucheton, B., Camara, M., Harris, A. *et al.* (2016) Tsetse control and the elimin-ation of Gambian sleeping sickness. *PLoS Neglected Tropical Diseases* 10, e0004437.

Mahamat, M.H., Peka, M., Rayaisse, J.B., Rock, K.S., Toko, M.A. *et al.* (2017) Adding tsetse control to med-ical activities contributes to decreasing transmission of sleeping sickness in the Mandoul focus (Chad). *PLoS Neglected Tropical Diseases* 11, e0005792.

Maltz, M.A., Weiss, B.L., O'Neill, M., Wu, Y. and Aksoy, S. (2012) OmpA-mediated biofilm formation is essential for the commensal bacterium *Sodalis glossinidius* to colonize the tsetse fly gut. *Applied and Environmental Microbiology* 78, 7760–7768.

Mbewe, N.J., Mweempwa, C., Guya, S. and Wamwiri, F.N. (2015) Microbiome frequency and their associ-ation with trypanosome infection in male *Glossina morsitans* centralis of Western Zambia. *Veterinary Parasitology* 211, 93–98.

McGraw, E.A. and O'Neill, S.L. (2013) Beyond insecticides: new thinking on an ancient problem. *Nature Reviews Microbiology* 11, 181–193.

Medina Munoz, M., Spencer, N., Enomoto, S., Dale, C. and Rio, R.V.M. (2020) Quorum sensing sets the stage for the establishment and vertical transmission of *Sodalis praecaptivus* in tsetse flies. *PLoS Genetics* 16, e1008992.

Michalkova, V., Benoit, J.B., Weiss, B.L., Attardo, G.M. and Aksoy, S. (2014) Vitamin B6 generated by obli-gate symbionts is critical for maintaining proline homeostasis and fecundity in tsetse flies. *Applied and Environmental Microbiology* 80, 5844–5853.

Nantulya, V.M. and Moloo, S.K. (1988) Suppression of cyclical development of *Trypanosoma brucei brucei* in *Glossina morsitans* centralis by an anti-procyclics monoclonal antibody. *Acta Tropica* 45, 137–144.

Ndung'u, J.M., Boulange, A., Picado, A., Mugenyi, A., Mortensen, A. *et al.* (2020) Trypa-NO! contributes to the elimination of *gambiense* human African trypanosomiasis by combining tsetse control with 'screen, diagnose and treat' using innovative tools and strategies. *PLoS Neglected Tropical Diseases* 14, e0008738.

O'Neill, S.L., Gooding, R.H. and Aksoy, S. (1993) Phylogenetically distant symbiotic microorganisms reside in *Glossina* midgut and ovary tissues. *Medical and Veterinary Entomology* 7, 377–383.

Pais, R., Lohs, C., Wu, Y., Wang, J. and Aksoy, S. (2008) The obligate mutualist *Wigglesworthia glossinidia* influences reproduction, digestion, and immunity processes of its host, the tsetse fly. *Applied and Environmental Microbiology* 74, 5965–5974.

Pontes, M.H. and Dale, C. (2006) Culture and manipulation of insect facultative symbionts. *Trends in Microbiology* 14, 406–412.

Rayaisse, J.B., Esterhuizen, J., Tirados, I., Kaba, D., Salou, E. *et al.* (2011) Towards an optimal design of target for tsetse control: comparisons of novel targets for the control of Palpalis group tsetse in West Africa. *PLoS Neglected Tropical Diseases* 5, e1332.

Rio, R.V., Hu, Y. and Aksoy, S. (2004) Strategies of the home-team: symbioses exploited for vector-borne disease control. *Trends in Microbiology* 12, 325–336.

Rio, R.V., Symula, R.E., Wang, J., Lohs, C., Wu, Y.N. *et al.* (2012) *Insight into the transmission biology and species-specific functional capabilities of tsetse (Diptera: Glossinidae) obligate symbiont Wigglesworthia.* mBio 3, e00240-11

Rose, C., Belmonte, R., Armstrong, S.D., Molyneux, G., Haines, L.R. *et al.* (2014) An investigation into the protein composition of the teneral *Glossina morsitans morsitans* peritrophic matrix. *PLoS Neglected Tropical Diseases* 8, e2691.

Rose, C., Casas-Sanchez, A., Dyer, N.A., Solorzano, C., Beckett, A.J. *et al.* (2020) *Trypanosoma brucei* colonizes the tsetse gut via an immature peritrophic matrix in the proventriculus. *Nature Microbiology* 5, 909–916.

Schneider, D.I., Saarman, N., Onyango, M.G., Hyseni, C., Opiro, R. *et al.* (2019) Spatio-temporal distribution of *Spiroplasma* infections in the tsetse fly (*Glossina fuscipes fuscipes*) in northern Uganda. *PLoS Neglected Tropical Diseases* 13, e0007340.

Shropshire, J.D., Leigh, B. and Bordenstein, S.R. (2020) Symbiont-mediated cytoplasmic incompatibility: what have we learned in 50 years? *Elife* 9, e61989.

Simarro, P.P., Diarra, A., Ruiz Postigo, J.A., Franco, J.R. and Jannin, J.G. (2011) The human African trypanosomiasis control and surveillance programme of the World Health Organization 2000–2009: the way forward. *PLoS Neglected Tropical Diseases* 5, e1007.

Simo, G., Kante, S.T., Madinga, J., Kame, G., Farikou, O. *et al.* (2019) Molecular identification of *Wolbachia* and *Sodalis glossinidius* in the midgut of *Glossina fuscipes quanzensis* from the Democratic Republic of Congo. *Parasite* 26, 5.

Snyder, A.K. and Rio, R.V. (2015) '*Wigglesworthia morsitans*' folate (vitamin B9) biosynthesis contributes to tsetse host fitness. *Applied and Environmental Microbiology* 81, 5375–5386.

Snyder, A.K., Deberry, J.W., Runyen-Janecky, L. and Rio, R.V. (2010) Nutrient provisioning facilitates homeostasis between tsetse fly (Diptera: Glossinidae) symbionts. *Proceedings of the Royal Society B* 277, 2389–2397.

Solano, P., Torr, S.J. and Lehane, M.J. (2013) Is vector control needed to eliminate *gambiense* human African trypanosomiasis? *Frontiers in Cellular and Infection Microbiology* 3, 33.

Son, J.H., Weiss, B.L., Schneider, D.I., Dera, K.M., Gstöttenmayer, F. *et al.* (2021) Infection with endosymbiotic *Spiroplasma* disrupts tsetse (*Glossina fuscipes fuscipes*) metabolic and reproductive homeostasis. *PLoS Pathogens* 17, e1009539.

Soumana, I.H., Simo, G., Njiokou, F., Tchicaya, B., Abd-Alla, A.M. *et al.* (2013) The bacterial flora of tsetse fly midgut and its effect on trypanosome transmission. *Journal of Invertebrate Pathology* 112 (Suppl.) S89–93.

Stijlemans, B., Conrath, K., Cortez-Retamozo, V., Van Xong, H., Wyns, L. *et al.* (2004) Efficient targeting of conserved cryptic epitopes of infectious agents by single domain antibodies. African trypanosomes as paradigm. *Journal of Biological Chemistry* 279, 1256–1261.

Symula, R.E., Alam, U., Brelsfoard, C., Wu, Y., Echodu, R. *et al.* (2013) *Wolbachia* association with the tsetse fly, *Glossina fuscipes fuscipes*, reveals high levels of genetic diversity and complex evolutionary dynamics. *BMC Evolutionary Biology* 13, 31.

Terenius, O., Marinotti, O., Sieglaff, D. and James, A.A. (2008) Molecular genetic manipulation of vector mosquitoes. *Cell Host & Microbe* 4, 417–423.

Toh, H., Weiss, B.L., Perkin, S.A., Yamashita, A., Oshima, K. *et al.* (2006) Massive genome erosion and functional adaptations provide insights into the symbiotic lifestyle of *Sodalis glossinidius* in the tsetse host. *Genome Research* 16, 149–156.

Turelli, M. and Hoffmann, A.A. (1995) Cytoplasmic incompatibility in *Drosophila simulans*: dynamics and parameter estimates from natural populations. *Genetics* 140, 1319–1338.

Turelli, M. and Hoffmann, A.A. (1999) Microbe-induced cytoplasmic incompatibility as a mechanism for introducing transgenes into arthropod populations. *Insect Molecular Biology* 8, 243–255.

Vale, G.A., Hargrove, J.W., Lehane, M.J., Solano, P. and Torr, S.J. (2015) Optimal strategies for controlling riverine tsetse flies using targets: a modelling study. *PLoS Neglected Tropical Diseases* 9, e0003615.

Vanhamme, L. and Pays, E. (2004) The trypanosome lytic factor of human serum and the molecular basis of sleeping sickness. *International Journal of Parasitology* 34, 887–898.

Vickerman, K. (1978) Antigenic variation in trypanosomes. *Nature* 273, 613–617.

Vreysen, M.J., Saleh, K.M., Ali, M.Y., Abdulla, A.M., Zhu, Z.R. *et al.* (2000) *Glossina austeni* (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique. *Journal of Economic Entomology* 93, 123–135.

Vreysen, M.J., Saleh, K., Mramba, F., Parker, A., Feldmann, U. *et al.* (2014) Sterile insects to enhance agricultural development: the case of sustainable tsetse eradication on Unguja Island, Zanzibar, using an area-wide integrated pest management approach. *PLoS Neglected Tropical Diseases* 8, e2857.

Wamwiri, F.N., Alam, U., Thande, P.C., Aksoy, E., Ngure, R.M. *et al.* (2013) *Wolbachia, Sodalis* and trypanosome co-infections in natural populations of *Glossina austeni* and *Glossina pallidipes*. *Parasites and Vectors* 6, 232.

Wang, J. and Aksoy, S. (2012) PGRP-LB is a maternally transmitted immune milk protein that influences symbiosis and parasitism in tsetse's offspring. *Proceedings of the National Academy of Sciences USA* 109, 10552–10557.

Wang, J., Wu, Y., Yang, G. and Aksoy, S. (2009) Interactions between mutualist *Wigglesworthia* and tsetse peptidoglycan recognition protein (PGRP-LB) influence trypanosome transmission. *Proceedings of the National Academy of Sciences USA* 106, 12133–12138.

Wang, S. and Jacobs-Lorena, M. (2013) Genetic approaches to interfere with malaria transmission by vector mosquitoes. *Trends in Biotechnology* 31, 185–193.

Weiss, B. and Aksoy, S. (2011) Microbiome influences on insect host vector competence. *Trends in Parasitology* 27, 514–522.

Weiss, B.L., Mouchotte, R., Rio, R.V., Wu, Y.N., Wu, Z. *et al.* (2006) Interspecific transfer of bacterial endosymbionts between tsetse fly species: infection establishment and effect on host fitness. *Applied and Environmental Microbiology* 72, 7013–7021.

Weiss, B.L., Wang, J. and Aksoy, S. (2011) Tsetse immune system maturation requires the presence of obligate symbionts in larvae. *PLoS Biology* 9, e1000619.

Weiss, B.L., Maltz, M. and Aksoy, S. (2012) Obligate symbionts activate immune system development in the tsetse fly. *Journal of Immunology* 188, 3395–3403.

Weiss, B.L., Wang, J., Maltz, M.A., Wu, Y. and Aksoy, S. (2013) Trypanosome infection establishment in the tsetse fly gut is influenced by microbiome-regulated host immune barriers. *PLoS Pathogens* 9, e1003318.

Weiss, B.L., Savage, A.F., Griffith, B.C., Wu, Y. and Aksoy, S. (2014) The peritrophic matrix mediates differential infection outcomes in the tsetse fly gut following challenge with commensal, pathogenic, and parasitic microbes. *Journal of Immunology* 193, 773–782.

Weiss, B.L., Maltz, M.A., Vigneron, A., Wu, Y., Walter, K.S. *et al.* (2019) Colonization of the tsetse fly midgut with commensal *Kosakonia cowanii* Zambiae inhibits trypanosome infection establishment. *PLoS Pathogens* 15, e1007470.

Welburn, S.C. and Maudlin, I. (1992) The nature of the teneral state in *Glossina* and its role in the acquisition of trypanosome infection in tsetse. *Annals of Tropical Medicine and Parasitology* 86, 529–536.

Welburn, S.C., Arnold, K., Maudlin, I. and Gooday, G.W. (1993) Rickettsia-like organisms and chitinase production in relation to transmission of trypanosomes by tsetse flies. *Parasitology* 107 (Pt 2), 141–145.

Welburn, S.C., Maudlin, I. and Molyneux, D.H. (1994) Midgut lectin activity and sugar specificity in teneral and fed tsetse. *Medical and Veterinary Entomology* 8, 81–87.

Werren, J.H., Baldo, L. and Clark, M.E. (2008) *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology* 6, 741–751.

Whitlow, M., Bell, B.A., Feng, S.L., Filpula, D., Hardman, K.D. *et al.* (1993) An improved linker for single-chain Fv with reduced aggregation and enhanced proteolytic stability. *Protein Engineering* 6, 989–995.

Xong, H.V., Vanhamme, L., Chamekh, M., Chimfwembe, C.E., Van Den Abbeele, J. *et al.* (1998) A VSG expression site-associated gene confers resistance to human serum in *Trypanosoma rhodesiense*. *Cell* 95, 839–846.

Yang, L., Weiss, B.L., Williams, A.E., Aksoy, E., de Silva Orfano, A. *et al.* (2021) Paratransgenic manipulation of a tsetse microRNA alters the physiological homeostasis of the fly's midgut environment. *PLoS Pathogens* 17, e1009475.

Zabalou, S., Riegler, M., Theodorakopoulou, M., Stauffer, C., Savakis, C. and Bourtzis, K. (2004). *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. *Proceedings of the National Academy of Sciences USA* 101, 15042–15045.

Zhao, X., Alves e Silva, T.L., Cronin, L., Savage, A.F., O'Neill, M. *et al.* (2015) Immunogenicity and serological cross-reactivity of saliva proteins among different tsetse species. *PLoS Neglected Tropical Diseases* 9, e0004038.

14 Paratransgenic Control of Chagas Disease

Ivy Hurwitz[1], Nicole Klein[2], Adam P. Forshaw[3], Ellen M. Dotson[4]* and Ravi V. Durvasula[5]

[1]*Center for Global Health, University of New Mexico Health Sciences Center, Albuquerque, New Mexico;* [2]*Sawtooth Epidemiology and Infectious Disease, Boise, Idaho;* [3]*Department of Anesthesiology, University of Wisconsin-Madison, Madison, Wisconsin;* [4]*Division of Parasitic Diseases and Malaria, Centers for Disease Control and Prevention, Atlanta, Georgia;* [5]*Infectious Diseases, Mayo Clinic, Jacksonville, Florida*

14.1 Introduction

Chagas disease, also known as American trypanosomiasis, is a vector-borne infection caused by the parasite *Trypanosoma cruzi*. Designated a 'neglected tropical disease' by the World Health Organization (WHO), Chagas disease is responsible for significant morbidity and mortality in Central and South America.

Chagas disease is most commonly transmitted to humans through the bite of its vector, the triatomine bug, which infests traditional human dwellings in endemic areas. Manifestations of the disease can be acute or chronic, and chronic infection is characterized by cardiac and gastrointestinal complications. Though usually thought of as a disease of endemic areas of Central and South America, recent surges in migration of humans from endemic to non-endemic areas has raised concern for undetected cases of chronic infection in non-endemic countries. In these non-endemic areas as well as endemic areas, *T. cruzi* can be transmitted through non-vectorial routes: congenitally (from infected mother to child) or through blood transfusion or organ donation from an infected individual. Treatment of Chagas disease has been plagued by poor tolerability and efficacy of medications in the chronic stage of disease as well as lack of access and availability of these medications.

Traditionally, in endemic areas, efforts to prevent human infection with *T. cruzi* have focused on education of rural populations and widespread pesticide application to control the triatomine vector of the parasite. Though some success has been achieved through multinational collaborative campaigns, inability to eliminate the massive sylvatic mammalian reservoir of the disease, instability of insecticides in the environment, development of pesticide resistance in triatomine bugs and chronic lack of funding for control programmes have all contributed to long-term failure to eliminate the disease. For these reasons, novel and innovative approaches to controlling vector-borne transmission of Chagas disease are being investigated.

*Corresponding author, email: edotson@cdc.gov

14.2 Chagas Disease

14.2.1 Epidemiology, ecology and modes of transmission of Chagas disease

In 1909 a Brazilian physician, Dr Carlos Chagas, described a parasite found in the hindgut of 'vinchucas' (*Triatoma infestans*), responsible for an outbreak of febrile illness in workers of the Central Railroad of Brazil. He named this parasite *Trypanosoma cruzi* and the disease became known as American trypanosomiasis, or Chagas disease.

In 2002, the WHO estimated the burden of Chagas disease in Latin America to be as high as 2.7 times the combined burden of malaria, schistosomiasis, leishmaniasis and leprosy (WHO, 2002). An estimated 6 million humans are infected in endemic areas of Latin America, and 70 million people are at risk of *T. cruzi* infection worldwide (PAHO, 2021). In 2013, the annual global burden in health care costs and disability-adjusted life years (DALYs) was estimated to be US$ 7.2 billion (Lee *et al.*, 2013). In 2019, despite widespread elimination campaigns, DALYs associated with Chagas disease in Latin America were estimated to be 275,000 (IHME, 2019).

Triatomine bugs (*Triatoma* spp., *Panstrongylus* spp., *Rhodnius* spp. and other genera), also known as 'kissing bugs', inhabit rural areas from the southern USA to the southern tip of Argentina. These insects may infest homes or can be completely sylvatic. A few species are sylvatic in some areas of their distribution but domiciliated in others, which has facilitated their elimination from the latter areas as is the case of *Triatoma infestans* in Brazil. After taking a bloodmeal from an infected mammalian host, the bug carries *T. cruzi* in the hindgut. Common mammalian reservoirs include dogs, rats, opossums, guinea pigs, skunks, armadillos, wood rats and raccoons. In endemic areas, triatomine bugs infest traditional mud-walled or thatch-roofed human dwellings, biting humans while they sleep and transmitting the parasite by defecating into the fresh bite wound.

As mentioned above, *T. cruzi* can also be transmitted by non-vectorial mechanisms. According to the US Centers for Disease Control and Prevention (CDC), congenital transmission and transmission through blood transfusions, organ transplantation, or laboratory accidents also occur (CDC, 2002). An estimated 5% of infected women will pass the parasite across the placenta to their fetus (Freilij and Altcheh, 1995; Gurtler *et al.*, 2003; Torrico *et al.*, 2004). Risk factors for congenital transmission include multiple pregnancies (Salas *et al.*, 2007), high maternal parasitaemia during acute infection, and low immune response to infection in mother or child (Hermann *et al.*, 2004).

Transfusion-associated *T. cruzi* infection has been reported in immunocompromised patients receiving whole-blood-derived platelets (Leiby *et al.*, 1999; CDC, 2007; Benjamin *et al.*, 2012). Infection acquired through solid organ transplantation has been reported in both Latin America and the USA (CDC, 2002, 2006).

In addition, oral transmission of disease by human consumption of food or drink contaminated with infected triatomine faeces or ground triatomine bugs has also been well documented (see review by Velasquez-Ortiz and Ramirez, 2020). Outbreaks associated with contaminated sugar-cane juice (Maguire *et al.*, 1986; Shikanai-Yasuda *et al.*, 1991; Benchimol-Barbosa, 2006), açaí palm juice and paste (Nobrega *et al.*, 2009), water (Dias *et al.*, 2008) and a regional fruit, bacaba (Pinto *et al.*, 2008), have been reported in Brazil. Contaminated guava juice caused an outbreak of Chagas disease in school children in Venezuela (Alarcon de Noya *et al.*, 2010).

14.2.2 Global spread of Chagas disease

In endemic areas, Chagas disease affects rural, economically disadvantaged, marginalized populations living in traditional mud–thatch housing infested with triatomine bugs. For these reasons, a social stigma of poverty, inability to work and

poor health is associated with the disease in endemic areas. This social stigma, along with the health disparity of Chagas disease in the population which it affects, led Hotez *et al.* (2012) to compare Chagas disease in Latin America with the early days of the HIV/AIDS epidemic in the USA. The socially marginalized populations affected by each disease (gay men in the early HIV/AIDS epidemic and poor rural populations in Chagas disease) often suffer from lack of access to adequate healthcare, compounded by fear of seeking healthcare due to social stigmas associated with their communities and their diagnoses. Furthermore, Hotez *et al.* noted that the chronicity of each disease requires continued medical monitoring and high cost of treatment over time. In addition, increased migration of humans from Chagas disease-endemic to non-endemic areas has led to a silent threat of transfusion-associated transmission of disease in non-endemic countries, resembling the threat of transfusion-associated HIV infection before widespread blood bank screening was instituted (Hotez *et al.*, 2012).

Recent studies of blood bank data from around the world have documented the rising seroprevalence of Chagas disease in non-endemic countries. As migration increases from Chagas endemic to non-endemic areas, global concern for a silent epidemic has been raised. Using blood bank data from countries receiving large numbers of Latin American immigrants, immigration data and known rates of disease in endemic countries, numbers of individuals infected with *T. cruzi* in non-endemic countries have been estimated. These estimates suggest > 340,000 infected individuals in the USA, > 5500 in Canada, > 100,000 in Europe (> 86,000 of these in Spain alone), > 3000 in Japan and > 3000 in Australia (Schmunis, 2007; Bern and Montgomery, 2009; Schmunis and Yadon, 2010; Manne-Goehler *et al.*, 2016). In these countries, where Chagas disease is not endemic, healthcare providers often lack experience in diagnosing the disease; education programmes for those at risk of passing on the infection are not in place; and access to healthcare for the immigrant segment of the population most often affected is a challenge.

14.3 Novel Approaches to Control of Chagas Disease

Given the issues of efficacy, toxicity and availability which plague the medical treatment of Chagas disease, efforts to decrease the global burden of the disease have traditionally focused on controlling the triatomine vector and public education to decrease risk of transmission. In the 1990s, several intergovernmental initiatives between endemic countries in Latin America developed multifaceted campaigns employing widespread insecticide use, improved housing conditions and promotion of public education aiming to decrease transmission of Chagas disease. The Pan American Health Organization (PAHO) reported that annual deaths attributed to Chagas disease fell from 45,000 in 1990 to 12,000 in 2020 (PAHO, 2021), and estimates of infections worldwide fell from 30 million in 1990 to 6 million in 2020 (PAHO, 2021). However, persistence of large sylvatic reservoirs of *T. cruzi* in mammalian hosts has prevented elimination of the disease (John and Hoppe, 1986). Surveillance data suggest a resurgence of human infections, particularly in Gran Chaco (Gurtler *et al.*, 2007), a large lowland plain in South America. In addition, large numbers of people with chronic Chagas disease can transmit the infection by blood donation, organ donation, or congenitally from mother to child. For these reasons, novel approaches to prevent transmission of *T. cruzi* to humans are being developed.

14.3.1 Paratransgenesis

The paratransgenic strategy (Fig. 14.1) has been described as the 'Trojan Horse' approach to controlling transmission of infectious disease. In this approach, a symbiotic bacterium within a given pathogen-transmitting vector is identified and genetically altered to produce molecules that kill the pathogen. The transformed bacterium is introduced into the insect, where expression of the transgene would interfere with pathogen differentiation
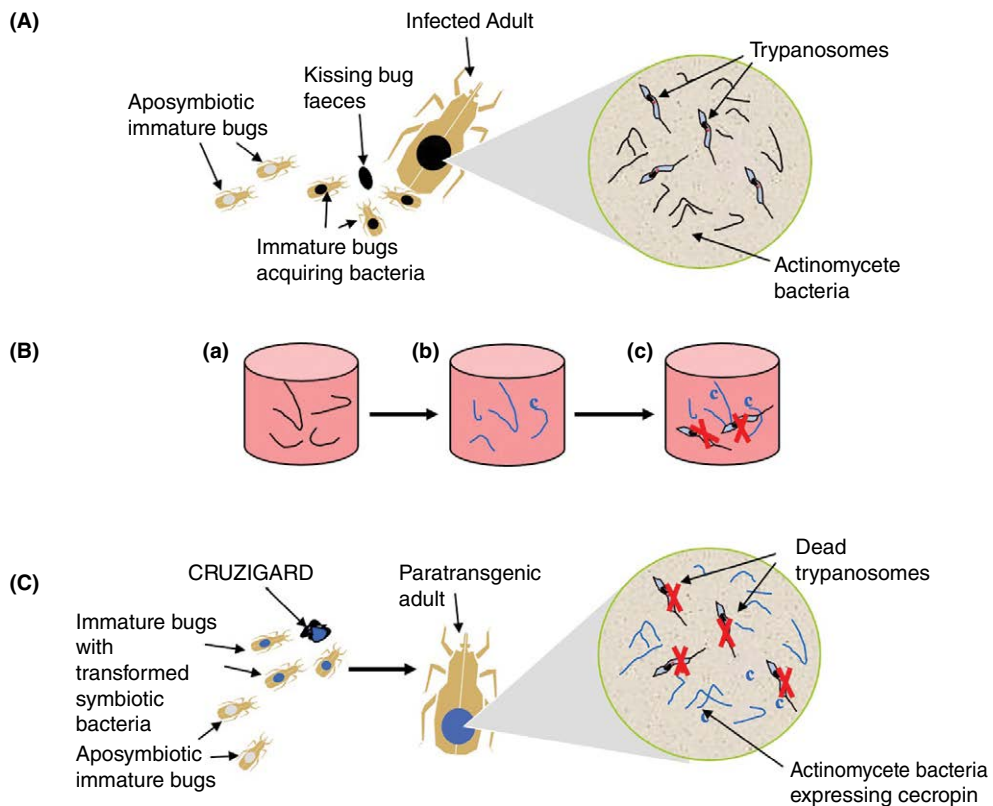
Fig. 14.1. Paratransgenesis in triatomines. (A) Triatomines harbour bacterial symbionts, such as the actinomycete *Rhodococcus rhodnii* which are essential for survival and reproduction, in their intestines where trypanosomes are found. Symbionts are passed to immature stages by coprophagy. **(B)** It is possible to **(a)** culture these symbionts and **(b)** genetically transform them to express a gene and release the peptide, in this case cecropin, into the medium **(c)**, which is detrimental to trypanosomes. Insects harbouring these bacteria would be incapable of transmitting Chagas disease. (C) Immature normal insects would feed on artificial faeces such as CRUZIGARD, a paste containing the genetically modified symbionts, guar gum and India ink, resulting in a population of vectors that can no longer transmit Chagas disease (paratransgenic insect).

or maturation. This disrupts the cycle of the infection, thereby abrogating the disease process. The use of the paratransgenic approach to control transmission of Chagas disease was first described by Beard *et al.* (1992).

Most, if not all, triatomine bugs are haematophagous, subsisting only on vertebrate bloodmeals. To supplement their basic nutritional and developmental needs, these insects have developed a symbiotic relationship with nocardiform actinomycetes (Baines, 1956). These bacteria are thought to aid in the processing of B-complex vitamins in the restricted blood diets of the host

and are essential to the survival of the triatomine. *Rhodococcus rhodnii* was identified in 1956 as a symbiont that lives extracellularly in the gut lumen of *Rhodnius prolixus*, the principal vector for Chagas disease in Central America (Baines, 1956). The vital role of *R. rhodnii* in the growth and development of *R. prolixus* has been demonstrated repeatedly under laboratory conditions. *Rhodnius prolixus* nymphs that lack gut-associated symbionts (aposymbiotic) do not reach sexual maturity and most will die after the second developmental moult. Introduction of the bacteria to first- or second-instar nymphs permits

normal growth and maturation (Baines, 1956; Harington 1960; Hill *et al.*, 1976).

We identified a novel *Corynebacterium* symbiont from laboratory colonies of *T. infestans* isolated from the Gran Chaco region of Argentina (Durvasula *et al.*, 2008) and studied the role of nocardiform actinomycetes such as *Rhodococcus* spp., *Nocardia* spp., *Gordonia* spp., *Corynebacterium* spp. and *Tsukumurella* (Durvasula *et al.*, 1997, 1999a, 2008; Beard *et al.*, 1998, 2002) in a number of triatomine bugs, including *Triatoma dimidiata* and *Triatoma sordida* (Pennington and Durvasula, unpublished data). We noted that absence of matched actinomycetes in the arthropod resulted in growth arrest and death of nymphs. However, when introduced into the normal vector, the actinomycetes supported growth and sexual maturation of these bugs, suggesting that symbionts are also highly specific to their respective hosts. In 1992, we transformed *R. rhodnii* with pRr1.1, a shuttle plasmid containing a gene encoding resistance to the antibiotic thiostrepton, to support the hypothesis that a transgene-carrying symbiont could be introduced into *R. prolixus*. We demonstrated that the modified symbiont can be maintained through the insect's development without adverse effects on insect survival and fitness (Beard *et al.*, 1992).

14.3.2 Antimicrobial peptides as effector molecules

Antimicrobial peptides (AMPs) are small highly conserved molecules that play an important role in innate immune defence (Zasloff, 2002; Ganz, 2003). They have been identified in many multicellular organisms and function as 'first-line' defence against invading microbes, including protozoans. AMPs are usually small amphipathic and highly basic molecules that can discriminate between host and bacterial membranes by charge and composition (Hale and Hancock, 2007). While most AMPs disrupt membranes of non-host cells, other modes of actions, including interfering with host metabolism, modulation of host immune response and targeting cytoplasmic components, have been reported (Hancock and Scott, 2000; Yang *et al.*, 2002; Zasloff, 2002).

We inserted the gene that encodes for the AMP cecropin A into the pRr1.1 shuttle plasmid and transformed it into *R. rhodnii*. Aposymbiotic *R. prolixus* nymphs were then populated with the cecropin A-expressing symbiont (Durvasula *et al.*, 1997). The paratransgenic insects were then allowed to engorge on *T. cruzi*-laden human blood until they reached sexual maturity. At the end of the experimental period, we found that hindgut contents from paratransgenic insects harbouring the cecropin A expressing *R. rhodnii* were either devoid of *T. cruzi* trypomastigotes (65%) or maintained markedly reduced titres of the parasite (35%) (Durvasula *et al.*, 1997). This study provided proof-of-concept for the paratransgenic strategy, demonstrating that the *in vivo* expression of an AMP from a genetically modified symbiont can significantly reduce carriage of the infectious parasite from the host vector.

We have reported on the *in vitro* activities of six AMPs selected from different insect sources to determine their differential toxicity profiles against host bacterial strains and *T. cruzi* parasites (Fieck *et al.*, 2010). In this work, we demonstrated that apidaecin, cecropin A, magainin II and melittin displayed high toxicity against *T. cruzi* ($LC_{100} < 10$ μM) compared with *R. rhodnii* (minimum bactericidal concentration > 100 μM) in single synthetic peptide treatment regimens. These AMPs were then employed in pairwise treatment protocols against *T. cruzi*. Dual peptide treatments of *T. cruzi* showed synergistic or additive effects between different AMPs resulting in increased toxicity over any single AMP treatment. For example, when administered alone to *T. cruzi*, apidaecin killed the parasite at the 10 μM dose, but when used in combination with melittin, magainin II or cecropin A, this AMP exhibited complete lethality to *T. cruzi* at 1.0 μM – a tenfold decrease in the necessary lethal concentration. While all combinations exhibited additive activity compared with single AMP treatments, synergistic activity was observed when magainin II was applied in combination with apidaecin or

melittin. *R. rhodnii* expressing cecropin A, apidaecin, melittin or magainin II has been generated. Lysates isolated from these transformants are biologically active against *T. cruzi* (Fieck and Durvasula, unpublished). These transformants should be introduced either individually, or in combination, into aposymbiotic triatomine nymphs to test the toxicity of products from single and dual peptide-carrying symbionts to *T. cruzi* in aposymbiotic *R. prolixus* nymphs. Based on the additive effects seen in our *in vitro* assays, we would expect to see substantial improvement in *T. cruzi* clearance in paratransgenic insects harbouring dual peptide symbionts. Further, we suspect that the use of AMPs in combination would reduce the development of peptide resistance in target *T. cruzi* populations.

14.3.3 Single-chain antibodies

Recombinant single-chain fragment variable (scFv) antibodies are fusion proteins containing the variable regions of the heavy and light chains of immunoglobulins. These regions are connected to one another with a short flexible linker (Gly4Ser) *n*=3–5, that permits the two protein domains to interact effectively with their corresponding antigen (Markiv *et al.*, 2011). Despite the absence of the constant regions, these proteins retain specificity to target antigens comparable to that of parent immunoglobulins. Because of their small size, scFvs can be cloned into expression plasmids and expressed from bacterial transformants. These molecules are therefore uniquely suited for use in the paratransgenic strategy.

To test the functionality of scFvs within the gut of *R. prolixus*, an expression shuttle plasmid coding the murine anti-progesterone antibody fragment rDB3 was constructed and transformed into *R. rhodnii* (Durvasula *et al.*, 1999a). Aposymbiotic *R. prolixus* nymphs were exposed to DB3-expressing *R. rhodnii* symbionts and allowed to develop with feeding on bloodmeals. Subsequent examination revealed that the rDB3 antibody fragment was synthesized by the transformed *R. rhodnii* and secreted into the gut lumen throughout the development of the nymphs to the adult stage. Protein extracts from the gut of paratransgenic *R. prolixus* bound progesterone, suggesting that the presence and activity of scFvs could be maintained in the environment of the insect gut (Durvasula *et al.*, 1999a). Similar studies using the *Corynebacterium* symbiont of *T. infestans* conducted with the same shuttle plasmid also showed progesterone binding activity (Durvasula *et al.*, 2008).

Progression from the paratransgenic system employing a marker scFv to one utilizing effector scFvs required the development of antibodies with strong binding affinities to the parasite. Cell-surface sialylated mucin-like glycoproteins play an important structural and biological role in the life cycle of *T. cruzi* (Acosta-Serrano *et al.*, 2001). For example, *T. cruzi* expresses a developmentally regulated sialidase, which is used for surface sialylation by a trans-sialidase mechanism (Weston *et al.*, 1999). Sialylation is thought to provide protection for *T. cruzi* from the innate immune responses. This large family of cell-surface sialylated mucin-like glycoproteins clearly play important structural and biological roles in the parasite's life cycle (Acosta-Serrano *et al.*, 2001) and are therefore excellent targets for scFv binding. In this design, scFvs that target *T. cruzi* surface proteins interfere with the physical contact between trypanosomes and the vector. This interference model predicts that the activity of the effector scFv molecules would be specific to parasite development and will elicit fewer negative effects on the vector or transformed symbiont.

The monoclonal antibodies B72.3 and CA19.9 bind to the sialyl-Tn and sialyl-(le)a antigens, respectively. We have demonstrated that these antibodies also bind to surface glycans of *T. cruzi* (Markiv *et al.*, 2011). Using these monoclonals as a template, synthetic DNA sequences of B72.3 and CA19.9 antibody variable domains in VH–VL orientation were generated. The monomeric red fluorescent protein (mRFP) derived from the red fluorescent protein cloned from the *Discosoma* coral, DsRed, was inserted as a rigid linker between the heavy

and light chain fragments. This modification conferred extra stability and fluorescence to the scFvs. Binding to fixed *T. cruzi* epimastigotes and fluorescent optical properties of these scFvs were demonstrated using confocal microscopy (Markiv *et al.,* 2011). The ability of these molecules to bind and inactivate live parasites is being investigated. Demeu *et al.* (2019) developed another single-change antibody ScFv-10D8 that adheres to the surface *T. cruzi* metacyclic trypomastigotes. They cloned the gene, fused it with a 6xHis tag and expressed it in *Escherichia coli*. Pre-incubation with this antibody with the trypomastigotes reduced their ability to invade cells and this antibody was proposed as a potential candidate for paratransgenic use.

Functional multimeric (and thus multivalent) single-chain assemblies produced by shortening the linker region between the heavy and light chain fragments may also prove to be useful. Expression of these highly specific multivalent antibodies that recognize and can crosslink key epitopes on the surface of *T. cruzi* may prevent parasite interactions with vector gut epithelia, thereby preventing development of infection in paratransgenic insects. We are also in the process of subcloning genes encoding these novel antibodies into our shuttle vector system for expression in *R. rhodnii*.

14.3.4   β-1-3-glucanase

The thick coat of mucin-like glycoproteins that covers the surface of *T. cruzi* is required by the parasites for attachment, and subsequent infection, in the triatomine vector (Cooper *et al.*, 1993). Many of these glycoproteins are developmentally regulated and have been proposed to play a role in the binding of the cell body and the flagellum of *T. cruzi* to the membrane surface of the vector gut, an integral step in *T. cruzi* maturation (Cooper *et al.*, 1993). *Arthrobacter luteus* lyticase is a complex endoglucanase consisting of β-1,3-glucanase and alkaline protease that degrades β-1,3 and 1-6 glycosidic linkages (Scott and Schekman, 1980).

We had shown that *A. luteus* lyticase, an endoglucanase complex consisting of β-1,3-glucanase and alkaline proteases, is very efficient in lysing *T. cruzi in vitro*, but is non-toxic to *R. prolixus* (Hurwitz and Durvasula, unpublished). The disruption of the glycoconjugates by endoglucanases could arrest parasite development in the vector and abort the transmission cycle. The cDNA encoding *A. luteus* β-1,3-glucanase was inserted into pRrExpA, our *E. coli/R.rhodnii* shuttle vector. The recombinant β-1,3-glucanase is biologically active and clears *T. cruzi* at low concentrations (Jose *et al.*, 2013), suggesting that it could potentially be used as another effector molecule for the paratransgenic control of Chagas disease.

14.3.5   Additional methods for bacterial modifications

In the above sections, the method used to introduce new genes into bacteria was limited to the use of episomally located plasmids. Newer and potentially more stable methods have come into practice in recent years. The use of the integrative elements of an L1 mycobacteriaphage proved to be a stable method for the modification of the actinomycete *R. rhodnii*, the symbiont of choice for *R. prolixus* (Dotson *et al.*, 2003). New molecular tools such as DNA assembly, recombineering and genome editing technologies such CRISPR/Cas9 have been adapted for use in actinomycetes and could provide more for more efficient engineering to express foreign DNAs in symbiotic bacteria (Palazzotto *et al.,* 2019).

14.4   From Bench Top to Field Trials

Deployment of genetically altered lines of bacteria to target naturally occurring field populations of triatomine bugs may have profound environmental consequences. To this end, we have developed a mathematical model predicting horizontal gene transfer (HGT) between genetically modified *R. rhodnii* and *Gordonia rubropertinctus*, a closely

related non-target Gram-positive actinomycete (Matthews *et al.*, 2011). The model treats HGT as a composite event whose probability is determined by the joint probability of gene transfer through the modalities of transformation, transduction and conjugation. Genes are represented in matrices, with the Monte Carlo method and Markov chain analysis used to simulate and evaluate environmental conditions. The model predicts an HGT frequency of less than 1.14×10^{-16} per 100,000 generations at the 99% certainty level. This predicted transfer frequency is less than the estimated average mutation frequency in bacteria, 10^{-1} per gene per 1000 generations. This suggests that, even if HGT were to occur between *R. rhodnii* and *G. rubropertinctus*, the transgene would likely not persist in the recipient organism, and that the likelihood of these unwanted events is low and severity of consequences is minimal.

Beyond reliance on low levels of HGT occurring within naturally occurring parameters, we have also begun the development and testing of a 'barrier' method of containment and dispersal for paratransgenic technologies. At present, the field of paratransgenic control strategies is still at the laboratory stage, with a pressing need for risk mitigation strategies to meet the Environmental Protection Agency (EPA) requirements. The current EPA guidelines on the proposed release of genetically modified organisms (GMOs) into new environments state that risk mitigation strategies must be implemented to ensure GMOs: (i) are contained to their specific environment of introduction; (ii) do not outcompete native species for resources or cause detriment to native habitats; and (iii) do not significantly contribute to foreign gene contamination (HGT) (US EPA, 2011).

Our current system demonstrates decreased HGT as discussed previously; however, over-competition and containment are more problematic. Previously it was calculated that roughly 10^{12} bacteria were needed to be suspended in a guar gum formulation (given the name 'CRUZIGARD') to coat only 25% of a single experimental domicile for paratransgenic acquisition (Durvasula *et al.*,

1997, 1999b). Thus, to extrapolate the number of bacteria needed to coat real-world houses for paratransgenic control, the number soars into the millions-of-trillions. At these numbers, environmental contamination is readily assured, thus containment of microbes until delivery to the insect gut becomes paramount.

We have proposed a microencapsulation strategy utilizing biologically derived polymers such as sodium alginates for delivery of transformed bacteria into the insect gut (Arora *et al.*, 2015). Alginates are chemically inert, stable organic polymers derived from alginic acid consisting of two distinct monomers, guluronic (G) and mannuronic (M) acid, that are linked in various ratios in varying polymer lengths (usually 40–100 kDa). When combined with a di-cationic salt, alginate polymers form insoluble crosslinked 'egg-box' aggregates in the form of a hydrogel whose properties can be manipulated based on the ratio of G to M as well as crosslinking di-cation selection (Bashan, 1986; Lamas *et al.*, 2001; Bashan *et al.*, 2002). In this strategy, engineered bacteria are encased within a three-dimensional biopolymer matrix where they are contained until ingested by the target organism (Bextine and Thorvilson, 2002). This process can be micronized to ensure that such baits are sufficiently small for target insect ingestion. The biopolymer selection and design dictates that if the capsule is ingested, pH gradients and enzymes within the gut cause the polymer to swell and digest, releasing the encapsulated bacteria (Lin *et al.*, 2005). If the capsule is not taken up, the polymer undergoes a prolonged biodegradation period, well exceeding the lifespan of the encapsulated bacteria and eventually releasing dead bacteria. The release and biodegradation dynamics of these polymers can be significantly 'tuned' based on polymer selection and/or inclusion of other biopolymers such as cellulose, methylcellulose, chitosan, gelatine, agar or polyacrylamide (Lee *et al.*, 2004). These microcapsules can also be significantly 'doped' or coated with various chemicals to accomplish secondary objectives like UV stabilization or heat tolerance (Hedimbi *et al.*, 2008).

Our initial experiments with encapsulation of *Pantoea agglomerans* demonstrated that transformed bacteria can be contained indefinitely under varying conditions (including water saturation and high salt concentration) depending on polymer and cross-linker selection (Arora *et al.,* 2018). We also demonstrated that *P. agglomerans* can be delivered into the foregut and midgut of the glassy winged sharpshooter *Homalodisca vitripennis* (GWSS) that was allowed to feed on grape vines painted with bacteria-containing microcapsules (Arora *et al.*, 2018). This strategy is easily adapted to suit *R. prolixus* by including microencapsulated *R. rhodnii* to the CRUZIGARD guar-gum matrix.

14.5   Conclusions

Though the epidemiology of Chagas disease has changed in recent years due to human migration, the mainstay for disease control in endemic regions still relies heavily on the use of chemical pesticides. The Southern Cone Initiative, a pesticide-based campaign undertaken by the governments of Argentina, Bolivia, Brazil, Chile, Paraguay, Uruguay and Peru, was launched in 1991 to control Chagas disease by elimination of the main vector, *T. infestans*, from domestic environments. In nations such as Brazil, Uruguay and Chile, results have been dramatic and new cases of Chagas disease have been virtually eliminated (Moncayo, 2003; Moncayo and Ortiz Yanine, 2006). However, concerns regarding environmental toxicity and adverse effect on human health are limiting the use of many chemical pesticides. The increasing emergence of insect resistance has further undermined the efficacy of these pesticides. Finally, the cost of repeated pesticide application is taking its toll on many of these nations. We describe the paratransgenic strategy as a potential alternative methodology to controlling Chagas disease transmission. In bench studies, we have demonstrated that we can dramatically decrease parasite carriage in triatomine bugs that harbour symbionts that have been genetically altered to express anti-trypanosomal molecules such as antimicrobial peptides. Other molecules, such as the endoglucanase β-1,3-glucanase and several highly specific scFvs, were added to our armamentarium of effector molecules for the paratransgenic control of *T. cruzi* transmission. To address the question of risk associated with field release of genetically modified bacteria, we tested a novel microencapsulation strategy utilizing biologically derived polymers for targeted delivery of genetically modified bacteria into the midgut of triatomine bugs. The paratransgenic approach does not aim to eliminate triatomine bugs. Rather, the overarching goal of this methodology is to modulate the insects' ability to transmit a parasite. This can serve as an alternative approach, or be used in conjunction with other methodologies, for control of Chagas disease in endemic regions of the world. Nevertheless, paratransgenic programmes have been stalled at the field-testing stage, despite published insect models for triatomine bugs. Regulatory constraints for field testing and funding considerations to conduct large-scale releases remain hurdles. Our team continues to pursue these long-range goals.

Acknowledgements

References

Acosta-Serrano, A., Almeida, I.C., Freitas-Junior, L.H., Yoshida, N. and Schenkman, S. (2001) The mucin-like glycoprotein super-family of *Trypanosoma cruzi*: structure and biological roles. *Molecular and Biochemical Parasitology* 114, 143–150.

Alarcon De Noya, B., Diaz-Bello, Z., Colmenares, C., Ruiz-Guevara, R., Mauriello, L. *et al.* (2010) Large urban outbreak of orally acquired acute Chagas disease at a school in Caracas, Venezuela. *Journal of Infectious Diseases* 201, 1308–1315.

Arora, A.K., Forshaw, A., Miller, T.A. and Durvasula, R. (2015) A delivery system for field application of paratransgenic control. *BMC Biotechnology* 15, 59–70.

Arora, A., Pesko, K., Quintero-Hernandez, V., Possani, L., Miller, T. and Durvasula, R.A. (2018) Paratransgenic strategy to block transmission of *Xylella fastidiosa* from the glassy-winged sharpshooter *Homalodiscus vitripennis*. *BMC Biotechnology* 18, 50.

Baines, S. (1956) The role of the symbiotic bacteria in the nutrition of *Rhodnius prolixus* (Hemiptera). *Journal of Experimental Biology* 33, 533–541.

Bashan, Y. (1986) Alginate beads as synthetic inoculant carriers for slow release of bacteria that affect plant growth. *Applied and Environmental Microbiology* 51, 1089–1098.

Bashan, Y., Hernandez, J.-P., Leyva, L. and Bacilio, M. (2002) Alginate microbeads as inoculant carriers for plant growth-promoting bacteria. *Biology and Fertility of Soils* 35, 359–368.

Beard, C.B., Mason, P.W., Aksoy, S., Tesh, R.B. and Richards, F.F. (1992) Transformation of an insect symbiont and expression of a foreign gene in the Chagas' disease vector *Rhodnius prolixus*. *American Journal of Tropical Medicine and Hygiene* 46, 195–200.

Beard, C.B., Durvasula, R.V. and Richards, F.F. (1998) Bacterial symbiosis in arthropods and the control of disease transmission. *Emergent Infectious Diseases* 4, 581–591.

Beard, C.B., Cordon-Rosales, C. and Durvasula, R.V. (2002) Bacterial symbionts of the triatominae and their potential use in control of Chagas disease transmission. *Annual Review of Entomology* 47, 123–141.

Benchimol-Barbosa, P.R. (2006) The oral transmission of Chagas' disease: an acute form of infection responsible for regional outbreaks. *International Journal of Cardiology* 112, 132–133.

Benjamin, R.J., Stramer, S.L., Leiby, D.A., Dodd, R.Y., Fearon, M. and Castro, E. (2012) *Trypanosoma cruzi* infection in North America and Spain: evidence in support of transfusion transmission. *Transfusion* 52, 1913–1921.

Bern, C. and Montgomery, S.P. (2009) An estimate of the burden of Chagas disease in the United States. *Clinical Infectious Diseases* 49, e52–54.

Bextine, B. and Thorvilson, H. (2002) Field applications of bait-formulated *Beauveria bassiana* alginate pellets for biological control of the red imported fire ant. *Environmental Entomology* 31, 746–752.

CDC (2002) Chagas disease after organ transplantation – United States 2001. *Morbidity and Mortality Weekly Report* 51, 210–212.

CDC (2006) Chagas disease after organ transplantation – Los Angeles, CA, 2006. *Morbidity and Mortality Weekly Report* 55, 798–800.

CDC (2007) Blood donor screening for Chagas disease – United States, 2006–2007. *Morbidity and Mortality Weekly Report* 56, 141–143.

Cooper, R., De Jesus, A.R. and Cross, G.A. (1993) Deletion of an immunodominant *Trypanosoma cruzi* surface glycoprotein disrupts flagellum-cell adhesion. *Journal of Cell Biology* 122, 149–156.

Demeu, L.M.K, Soares, R.J. Miranda, J.S. Pancheco-Lugo, L.A., Oliveira, K.G. *et al.* (2019) Engineering a single-chain antibody against *Trypanosoma cruzi* metacyclic trypomastigotes to block cell division. *PLoS ONE* 14(10), e0223773.

Dias, J.P., Bastos, C., Araujo, E., Mascarenhas, A.V., Martins Netto, E. *et al.* (2008) Acute Chagas disease outbreak associated with oral transmission. *Revista da Sociedade Brasileira de Medicina Tropical* 41, 296–300.

Dotson, E.M., Plikaytis, B., Shinnick T.M., Durvasula, R.V. and Beard, C.B. (2003) Transformation of *Rhodococcus rhodnii*, a symbiont of the Chagas disease vector *Rhodnius prolixus*, with integrative elements of the L1 mycobacteriophage. *Infection Genetics and Evolution* 3, 102–109.

Durvasula, R.V., Gumbs, A., Panackal, A., Kruglov, O., Aksoy, S. *et al.* (1997) Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. *Proceedings of the National Academy of Sciences USA* 94, 3274–3278.

Durvasula, R.V., Gumbs, A., Panackal, A., Kruglov, O., Taneja, J. *et al.* (1999a) Expression of a functional antibody fragment in the gut of *Rhodnius prolixus* via transgenic bacterial symbiont *Rhodococcus rhodnii*. *Medical and Veterinary Entomology* 13, 115–119.

Durvasula, R.V., Kroger, A., Goodwin, M., Panackal, A., Kruglov, O. *et al.* (1999b) Strategy for introduction of foreign genes into field populations of Chagas disease vectors. *Annals Entomology Society of America* 92, 937–943.

Durvasula, R.V., Sundaram, R.K., Kirsch, P., Hurwitz, I., Crawford, C.V. *et al.* (2008) Genetic transformation of a Corynebacterial symbiont from the Chagas disease vector *Triatoma infestans*. *Experimental Parasitology* 119, 94–98.

Fieck, A., Hurwitz, I., Kang, A.S. and Durvasula, R. (2010) *Trypanosoma cruzi:* synergistic cytotoxicity of multiple amphipathic anti-microbial peptides to *T. cruzi* and potential bacterial hosts. *Experimental Parasitology* 125, 342–347.

Freilij, H. and Altcheh, J. (1995) Congenital Chagas' disease: diagnostic and clinical aspects. *Clinical Infectious Diseases* 21, 551–555.

Ganz, T. (2003) Defensins: antimicrobial peptides of innate immunity. *Nature Reviews: Immunology* 3, 710–720.

Guhl, F., Pinto, N. and Aguilera, G. (2009) Sylvatic triatominae: a new challenge in vector control transmission. *Memorias do Instituto Oswaldo Cruz* 104 (Suppl. 1), 71–75.

Gurtler, R.E., Segura, E.L. and Cohen, J.E. (2003) Congenital transmission of *Trypanosoma cruzi* infection in Argentina. *Emerging Infectious Diseases* 9, 29–32.

Gurtler, R.E., Kitron, U., Cecere, M.C., Segura, E.L. and Cohen, J.E. (2007) Sustainable vector control and management of Chagas disease in the Gran Chaco, Argentina. *Proceedings of the National Academy of Sciences USA* 104, 16194–16199.

Hale, J.D. and Hancock, R.E. (2007) Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. *Expert Review of Anti-Infective Therapy* 5, 951–959.

Hancock, R.E. and Scott, M.G. (2000) The role of antimicrobial peptides in animal defenses. *Proceedings of the National Academy of Sciences USA* 97, 8856–88561.

Harington, J.S. (1960) Studies on *Rhodnius prolixus*: growth and development of normal and sterile bugs, and the symbiotic relationship. *Parasitology* 50, 279–286.

Hedimbi, M., Kaaya, G.P., Singh, S., Chimwamurombe, P.M., Gindin, G. *et al.* (2008) Protection of *Metarhizium anisopliae* conidia from ultra-violet radiation and their pathogenicity to *Rhipicephalus evertsi evertsi* ticks. *Experimental and Applied Acarology* 46, 149–156.

Hermann, E., Truyens, C., Alonso-Vega, C., Rodriguez, P., Berthe, A. *et al.* (2004) Congenital transmission of *Trypanosoma cruzi* is associated with maternal enhanced parasitemia and decreased production of interferon-gamma in response to parasite antigens. *Journal of Infectious Diseases* 189, 1274–1281.

Hill, P, Campbell, J.A. and Petrie, I.A. (1976) *Rhodnius prolixus* and its symbiotic actinomycete: a microbiological, physiological and behavioural study. *Proceedings of the Royal Society of London, B: Biological Sciences* 194, 501–525.

Hotez, P.J., Dumonteil, E., Woc-Colburn, L., Serpa, J.A., Bezek, S. *et al.* (2012) Chagas disease: 'the new HIV/AIDS of the Americas'. *PLoS Neglected Tropical Diseases* 6, e1498.

IHME (2019) Institute of Health Metrics and Evaluation. Chaga disease – Level 3 cause. Available at: www. Healthdata.org/resulst/gbd_summaries/2019/chaga-disease-level-e-cause (accessed 4 June 2021)

John, D.T. and Hoppe, K.L. (1986) *Trypanosoma cruzi* from wild raccoons in Oklahoma. *American Journal of Veterinary Research* 47, 1056–1059.

Jose, C., Klein, N., Wyss, S., Fieck, A., Hurwitz, I. and Durvasula, R. (2013) Recombinant *Arthrobacter* beta-1, 3-glucanase as a potential effector molecule for paratransgenic control of Chagas disease. *Parasite and Vectors* 6, 65.

Lamas, M.C., Bregni, C., Daquino, M., Degrossi, J. and Firenstein, R. (2001) Calcium alginate microspheres of *Bacillus subtilis*. *Drug Development and Industrial Pharmacy* 27, 825–829.

Lee, B.Y., Bacon, K.M., Bottazzi, M.E. and Hotez, P.J. (2013) Global economic burden of Chagas disease: a computational simulation model. *Lancet Infectious Disease* 13(4), 342–348.

Lee, J.S., Cha, D.S. and Park, H.J. (2004) Survival of freeze-dried *Lactobacillus bulgaricus* KFRI 673 in chitosan-coated calcium alginate microparticles. *Journal of Agricultural and Food Chemistry* 52, 7300–7305.

Leiby, D.A., Lenes, B.A., Tibbals, M.A. and Tames-Olmedo, M.T. (1999) Prospective evaluation of a patient with *Trypanosoma cruzi* infection transmitted by transfusion. *New England Journal of Medicine* 341, 1237–1239.

Lin, W.C., Yu, D.G. and Yang, M.C. (2005) pH-sensitive polyelectrolyte complex gel microspheres composed of chitosan/sodium tripolyphosphate/dextran sulfate: swelling kinetics and drug delivery properties. *Colloid Surface B* 44, 143–151.

Maguire, J.H., Hoff, R., Sleigh, A.C., Mott, K.E., Ramos, N.B. and Sherlock, I.A. (1986) An outbreak of Chagas' disease in southwestern Bahia, Brazil. *American Journal of Tropical Medicine and Hygiene* 35, 931–936.

Manne-Goehler, J., Umeh, C.A., Montgomery, S.P. and Wirtz, V.J. (2016) Estimating the burden of Chagas disease in the United States. *PLoS Neglected Tropical Diseases* 10(11), e0005033.

Markiv, A., Anani, B., Durvasula, R.V. and Kang, A.S. (2011) Module based antibody engineering: a novel synthetic RED antibody. *Journal of Immunological Methods* 364, 40–49.

Matthews, S., Sreehari Rao, V. and Durvasula, R.V. (2011) Modeling horizontal gene transfer (HGT) in the gut of the Chagas disease vector *Rhodnius prolixus*. *Parasite and Vectors* 4, 77.

Moncayo, A. (2003) Chagas disease: current epidemiological trends after the interruption of vectorial and transfusional transmission in the Southern Cone countries. *Memorias do Instituto Oswaldo Cruz* 98, 577–591.

Moncayo, A. and Ortiz Yanine, M.I. (2006) An update on Chagas disease (human American trypanosomiasis). *Annals of Tropical Medicine and Parasitology* 100, 663–677.

Nobrega, A.A., Garcia, M.H., Tatto, E., Obara, M.T., Costa, E. *et al.* (2009) Oral transmission of Chagas disease by consumption of acai palm fruit, Brazil. *Emerging Infectious Diseases* 15, 653–655.

PAHO (2021) Chagas disease. Pan American Health Organization. Available at www.paho.ort/en/topics/chagas-disease (accessed 4 June 2021)

Palazzotto, E., Tong, Y., Lee, S.Y. and Weber, T. (2019) Synthetic biology and metabolic engineering of actinomycetes for natural product discovery. *Biotechnology Advances* 37, 107366.

Pinto, A.Y., Valente, S.A., Valente Vda, C., Ferreira Junior, A.G. and Coura, J.R. (2008) [Acute phase of Chagas disease in the Brazilian Amazon region: study of 233 cases from Para, Amapa and Maranhao observed between 1988 and 2005]. *Revista da Sociedade Brasileira de Medicina Tropical* 41, 602–614.

Salas, N.A., Cot, M., Schneider, D., Mendoza, B., Santalla, J.A. *et al.* (2007) Risk factors and consequences of congenital Chagas disease in Yacuiba, south Bolivia. *Tropical Medicine and International Health* 12, 1498–1505.

Schmunis, G.A. (2007) Epidemiology of Chagas disease in non-endemic countries: the role of international migration. *Memorias do Instituto Oswaldo Cruz* 102 (Suppl. 1), 75–85.

Schmunis, G.A. and Yadon, Z.E. (2010) Chagas disease: a Latin American health problem becoming a world health problem. *Acta Tropica* 115, 14–21.

Scott, J.H. and Schekman, R. (1980) Lyticase: endoglucanase and protease activities that act together in yeast cell lysis. *Journal of Bacteriology* 142, 414–423.

Shikanai-Yasuda, M.A., Marcondes, C.B., Guedes, L.A., Siqueira, G.S., Barone, A.A. *et al.* (1991) Possible oral transmission of acute Chagas' disease in Brazil. *Revista da Sociedade Brasileira de Medicina Tropical* 33, 351–357.

Torrico, F., Alonso-Vega, C., Suarez, E., Rodriguez, P., Torrico, M.C. *et al.* (2004) Maternal *Trypanosoma cruzi* infection, pregnancy outcome, morbidity, and mortality of congenitally infected and non-infected newborns in Bolivia. *American Journal of Tropical Medicine and Hygiene* 70, 201–209.

US EPA (2011) Introduction to Biotechnology Regulation for Pesticides. US Environmental Protection Agency. Available at: http://www.epa.gov/oppbppd1/biopesticides/regtools/biotech-reg-prod.htm (accessed 27 April 2014)

Velasquez-Ortiz, N and Ramirez, J.D. (2020) Understanding the oral transmission of *Trypanosoma cruzi* as a veterinary and medical foodborne zoonosis. *Research in Veterinary Science* 132, 448–461.

Weston, D., Patel, B. and Van Voorhis, W.C. (1999) Virulence in *Trypanosoma cruzi* infection correlates with the expression of a distinct family of sialidase superfamily genes. *Molecular and Biochemical Parasitology* 98, 105–116.

WHO Expert Committee on the Control of Chagas Disease (2002) *Control of Chagas Disease*. Second report of the WHO Expert Committee. Brasilia, Brazil.

Yang, D., Biragyn, A., Kwak, L.W. and Oppenheim, J.J. (2002) Mammalian defensins in immunity: more than just microbicidal. *Trends in Immunology* 23, 291–296.

Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. *Nature* 415, 389–395.

15 *Asaia* Paratransgenesis in Mosquitoes

Maria Vittoria Mancini[1] and Guido Favia[2]*

[1]*Polo d'Innovazione di Genomica, Genetica e Biologia, Terni, Italy;* [2]*School of Biosciences & Veterinary Medicine - University of Camerino, Camerino (MC), Italy*

15.1 Asaia

Bacteria of the genus *Asaia* (family Acetobacteraceae) were described for the first time 20 years ago with the identification and isolation of *Asaia bogorensis* from Indonesian flowers of orchid tree (*Bauhinia purpurea*), plumbago (*Plumbago auriculata*) and fermented glutinous rice (Yamada *et al.*, 2000). Like other acetic acid bacteria, this anaerobic rod-shaped and peritrichously flagellated bacterium proliferates at low pH (3.0) and 30°C is considered its optimal growth temperature. It is able to oxidize acetate and lactate to carbon dioxide and water, and to produce acids from D-glucose, D-fructose, L-sorbose, dulcitol and glycerol (Yamada *et al.*, 2000); it is unable, however, to oxidize ethanol to acetic acid (Crotti *et al.*, 2009). It has also shown resistance to several antibiotics, including ceftazidime, meropenem, aztreonam, penicillin and ampicillin (Moore *et al.*, 2002). One year after its discovery, another species of this genus was isolated from a crown flower (dok rak, *Calotropis gigantea*) collected in Bangkok and was identified as *Asaia siamensis* (Katsura *et al.*, 2001).

The repertoire of isolates of *Asaia* increased over the years: *A. krungthepensis, A. lannaensis, A. platycodi, A. prunellae, A. astilbes* and *A. spathodeae* were included in the species array of this bacterial genus (Yukphan *et al.*, 2004; Suzuki *et al.*, 2010). The majority of the identified species were isolated from specimens of flowers collected in Japan, suggesting that the ecology of *Asaia* species is not only restricted to tropical regions. *Asaia* was also found to be associated with wine grapes, including industrial processes of wine production. In particular, one study reported the presence of this bacterium in wine grapes cultivated in vineyards located in New South Wales, Australia (Bae *et al.*, 2006). Another study, aiming to characterize bacterial populations involved in malolactic fermentation of Spanish Tempranillo wine, identified *Asaia* as a component of the natural bacterial community of this black grape variety (Ruiz *et al.*, 2010). Within the food industry, due to its strong adhesion ability and propensity to produce resistant biofilms, *Asaia* has been often recognized as a spoilage contaminant of non-alcoholic, non-carbonated beverages (Moore *et al.*, 2002).

---

*Corresponding author, email: guido.favia@unicam.it

Its association with insect species was initially demonstrated in the main Asian malaria vector, the mosquito *Anopheles stephensi* (Favia *et al*., 2007), and in the hemipteran *Scaphoideus titanus*, the leafhopper vector of the phytoplasma and the causative agent of *flavescence dorée*, one of the most relevant diseases of the grapevine (Marzorati *et al*., 2006). Isolates of *Asaia* from several insect species are listed in Table 15.1.

Since then, the symbiotic association between *Asaia* and insect species has been extended to several other mosquito species, including the major vectors of parasites and arboviruses in many regions of the world (*Anopheles gambiae*, *An. arabiensis*, *An. funestus*, *An. darlingi*, *An. maculipennis*, *Aedes aegypti*, *Ae. albopictus*, *Culex pipens* and *Cx. quinquefasciatus)* (Crotti *et al*., 2009; Chouaia *et al*., 2010; Damiani *et al*., 2010; Zouache *et al*., 2011; Epis *et al*., 2012b; De Freece *et al*., 2014; Alonso *et al*., 2019). Molecular and imaging studies localized *Asaia* in different mosquito organs, such as midgut, crop, salivary glands and reproductive organs of female and male individuals of *An. stephensi*, where it reaches 100% prevalence in laboratory colonies (Favia *et al*., 2007; Crotti *et al*.,

2009; Damiani *et al*., 2010). Furthermore, next-generation sequence (NGS) studies profiling the microbiota hosted in tissues of different mosquito species identified *Asaia* as a member of the natural microbiota of gut, reproductive organs and salivary glands of laboratory-reared populations of *Anopheles* and *Aedes* spp. (Mancini *et al*., 2018). Its association is not limited to mosquitoes: bacterial profiling of different insect pests revealed its presence in the brown planthopper *Nilaparvata lugens* (Tang *et al*., 2010), the cabbage white butterfly *Pieris rapae* (Robinson *et al*., 2010) and, more recently, in the Mediterranean fly *Ceratitis capitata* (Comandatore *et al*., 2021).

As mentioned above, several *Asaia* strains were isolated from tropical flowers, likely associated with phytotelmata, which are small cavities typical of terrestrial plants for water impoundment, commonly identified as natural breeding sites for some species of mosquito larvae (Mangudo *et al*., 2015; Roux and Robert, 2019). Therefore, it is possible to hypothesize that phytotelmata could represent the route of *Asaia* uptake during mosquito aquatic stages. Alternatively, mosquito adults can acquire *Asaia*

Table 15.1. List of *Asaia* strains isolated from insect species.

Host genus	Host species	Transmitted diseases/effects	Reference
Anopheles	*gambiae*	Malaria	Favia *et al*., 2007
	arabiensis		ibid.
	funestus		ibid.
	stephensi		ibid.
	maculipennis		ibid.
	darlingi		Alonso *et al*., 2019
Aedes	*aegypti*	Viral diseases	Crotti *et al*., 2009
	albopictus		Chouaia *et al*., 2010
	koreicus		Comandatore *et al*., 2021
	japonicus		ibid.
Culex	*quinquefasciatus*	Viral diseases	De Freece *et al*., 2014
	pipiens		ibid.
Lutzomya	*longipalpis*	Leishmaniasis	Akhoundi *et al*., 2012
Ceratitis	*capitata*	Fruit degradation	Comandatore *et al*., 2021
Bactrocera	*tryoni*	Fruit/vegetables degradation	Woruba *et al*., 2019
Scaphoideus	*titanus*	*Flavescence-dorée*	Crotti *et al*., 2009
Sogatella	*furcifera*	SRBSD virus	Li *et al*., 2020
Nilaparvata	*lugens*	Rice degradation	Tang *et al*., 2010
Pieris	*rapae*	Cabbage degradation	Robinson *et al*., 2010

while feeding on flower nectar. In fact, soon after eclosion, adults need to feed rapidly on sugar sources and *Asaia* cells contained in the nectar can be transferred directly to mosquito crop and midguts, where it is known to proliferate (Barredo and DeGennaro, 2020).

As detailed in the following, studies on the symbiotic traits of *Asaia*–host association suggest that *Asaia* has a beneficial, although not primary, role in host biology. Due to the nature of this type of association, insects and their symbionts have evolved a diverse array of structures and strategies to interact. Numerous examples of bacterial symbiosis show how variable the degree of dependence between the symbiont and its host could be. Usually this variation depends on how advantageous the associations are and drives co-evolutionary adaptations. Comparative genomics analysis of *Asaia* lineages from mosquito species belonging to *Anopheles* and *Aedes* genera displayed substantial variations in the genomic architecture among isolates (Alonso *et al.*, 2019). The latter study showed that one bacterial strain isolated from field-collected *Anopheles darlingi*, the main South American malaria vector, underwent a significant reduction in size and in gene content, compared with other mosquito-isolated strains. These preliminary findings were further strengthened and expanded by a comparative phylogenomics study revealing evidence of independent genome erosion processes across different *Asaia* lineages that occurred through a common similar pattern (Comandatore *et al.*, 2021). This variation suggests that *Asaia*, together with other rare known examples of bacteria, such as *Coxiella* and *Serratia*, represents a unique example of the genome-reduction phenomenon not only within a single bacterial lineage, but also between symbiotically associated isolates within highly related hosts (Gottlieb *et al.*, 2015; Manzano-Marìn and Latorre, 2016; Alonso *et al.*, 2019).

Within the past 15 years a large body of experimental data has been generated characterizing *Asaia* bacteria and the interplay with the insect host. Here we collate and describe these promising features in relation to the potential involvement of this symbiont in vector control strategies (Fig. 15.1).

15.2 Paratransgenesis for Vector Control

The evidence of the strong and pervasive ecological association between *Asaia* and many different mosquito species appears particularly promising in the frame of the paratransgenic control of malaria and other mosquito-borne diseases (Jacobs-Lorena, 2009). The strategy of paratransgenesis involves the genetic engineering of a microorganism within the insect host. Related to malaria paratransgenesis, bacteria inhabiting the mosquito gut could play a key role since the most vulnerable stage of *Plasmodium* development in the mosquito occurs within this organ. A small proportion of ingested gametocytes contained in an infected bloodmeal develops into ookinetes, and a further smaller fraction will develop to oocysts. It has been proven that more than 80% of the sporozoites circulating in the haemocoel fail to reach the salivary glands, being removed from the haemolymph through multiple mechanisms, including lytic and melanization events (Taylor, 1999). The oocyst stage is therefore a developmental bottleneck during the parasite life cycle; consequently, the co-localization of this parasite stage and the symbiont in the midgut represents a favourable event for developing site-specific paratransgenic interventions. Paratransgenic approaches can also target other organs of mosquitoes, for example the salivary glands, which represent the ultimate site of the parasite cycle within the invertebrate host.

The first demonstration of the feasibility of paratransgenic approaches to tackle vector-borne diseases was achieved with *Rhodococcus rhodnii*, a bacterial symbiont of the reduviid bug *Rhodnius prolixus*, the vector of Chagas disease (see Hurwitz *et al.*, Chapter 14, this volume) (Durvasula *et al.*, 1997; Taracena *et al.*, 2015). This bacterial symbiont was manipulated to express anti-parasite effector molecules and, once reintroduced in the vector population, showed strong inhibitory effects on the transmission of *Trypanosoma cruzi* (see Hurwitz *et al.*, Chapter 14, this volume) (Hurwitz *et al.*, 2011). Similar approaches were also exploited for the control of African trypanosomiasis
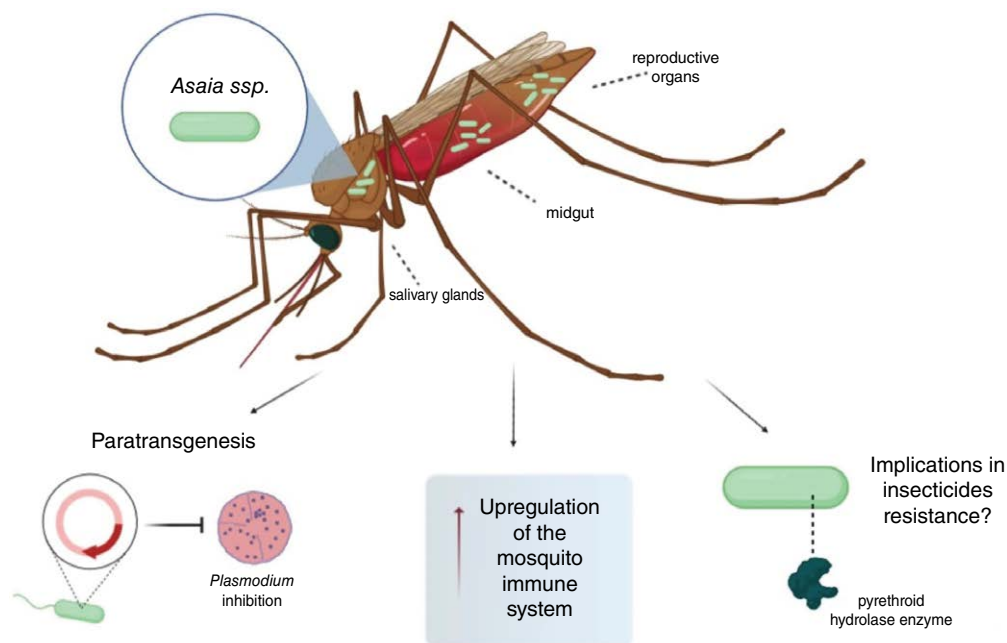
Fig. 15.1. ***Asaia* distribution in the mosquito.** *Asaia* is widely distributed in the salivary glands, midgut and reproductive organs, with some potential applications within insect hosts, including paratransgenic modifications for interfering with pathogen transmission, upregulation of the mosquito immune system and involvement in host insecticide resistance.

(De Vooght *et al*., 2012), leishmaniasis (Hurwitz *et al*., 2011) and malaria (see Guido *et al*., Chapter 16, this volume) (Wang *et al*., 2017). This approach was also extended to other insect pests, such as the sharpshooter *Homalodisca coagulata*, the vector of Pierce's crop disease (Bextine *et al*., 2004; Aksoy *et al*., 2008) and, more recently, the black bean aphid (Elston *et al*., 2021).

15.3 Desirable Attributes of *Asaia* as a Paratransgenic Candidate

Asaia possesses several key requirements for being considered as a paratransgenic candidate.

Firstly, it can be cultivated and propagated easily outside its host in cell-free media, and it is suitable for genetic transformation with exogenous DNA to produce recombinant proteins. Soon after its discovery in mosquitoes, *Asaia* transformability was tested by electroporating cells with replicative plasmids containing a green fluorescent protein (GFP) cassette, in order to make bacteria localization easier (Favia *et al*., 2007). Colonization of mosquitoes with GFP-expressing *Asaia* strains provided relevant insights on the symbiont's tissue tropism as well as its transstadial and transgenerational transmission routes. Results from fluorescence analysis of mosquitoes recolonized with GFP–*Asaia* via sugar meals confirmed the initial data from molecular analysis, showing that the bacterium efficiently colonizes the gut, salivary glands and male reproductive system (Favia *et al*. 2007; Damiani *et al*., 2008, 2010; Crotti *et al*., 2009). Additional studies proved that *Asaia* cells are spreading among *An. stephensi* populations by exploring both horizontal and vertical routes. The horizontal transmission occurs by mating and co-feeding within the same sugar source, while the vertical transmission occurs through maternal and paternal contributions (Favia *et al*., 2007, 2008; Damiani

et al., 2008, 2010). Evidence suggests that maternal transmission relies on the mechanism of egg-smearing: *Asaia* cells peripherally colonize the surfaces of embryos in female oocytes. Once emerged from laid eggs, larvae feed directly on eggshells, acquiring also their microbiota (Damiani *et al.*, 2010). This transmission mechanism was also reported in other insect species (Flórez *et al.*, 2017). Since the infection could theoretically be expanded to the whole larval population in the same breeding site, this transmission dynamic offers an interesting applicative approach for spreading recombinant bacteria in the field.

The detection of *Asaia* cells in male reproductive organs gave rise to the idea of investigating the paternal contribution in *Asaia* transfer. Damiani *et al.* (2008) showed that male-borne *Asaia* are transferred to females during mating in *An. stephensi* mosquitoes and they are also vertically transmitted to the progeny through egg smearing. In addition, *Asaia* transstadial transmission was demonstrated by tracking the spread of fluorescently marked *Asaia* from larvae to pupae and from pupae to adults (De Freece *et al.*, 2014).

In addition to the release of bacteria in natural mosquito breeding sites, the release of paratransgenic non-biting mosquito males in a specific area was theorized. This strategy looks particularly promising, since it overcomes the plausible ethical objections related to the release of biting females in the wild. An additional potential approach for distributing and selecting recombinant strains of *Asaia* expressing anti-pathogen molecules could be the use of 'feeding stations', consisting of locations where mosquitoes are attracted to sugar-based solutions spiked with recombinant *Asaia* cells. The feasibility of this approach is supported by direct field assessments and a very similar method used to distribute insecticides to wild mosquito populations (Müller *et al.*, 2010; Bilgo *et al.*, 2018).

The release of *Asaia*-carrying males and the use of *Asaia*-infected feeding stations in specific areas were tested as potential systems for *Asaia* dissemination in the environment. Trials in large cages hosting populations of thousands of mosquitoes and mimicking

natural field conditions (light, temperature and humidity diurnal cycles, behavioural stimuli) were used for assessing the ability of modified *Asaia* cells to invade and spread in wild-type populations of *An. stephensi* and *An. gambiae*. High rates of dissemination of modified bacteria in both malaria vectors were observed, despite the system of introduction of bacteria in the populations (Mancini *et al.*, 2016).

The high prevalence and almost complete vertical and horizontal transmission rates demonstrated in large cages trials is a promising element for an *Asaia*-based paratransgenesis approach since it allows the perpetuation of the infection by recombinant strains through generations, ensuring a self-spreading mechanism, which appears very efficient in field-like laboratory settings (Mancini *et al.*, 2016).

For several years, genetic transformation of *Asaia* has focused on the expression of fluorescent markers for understanding its tropism and transmission dynamics. However, an applicable paratransgenic symbiotic strain should be able to inhibit/reduce pathogen development. In order to be effective, the cargo effector molecules must be expressed and secreted within a specific time window and location in the insect vector in relation to the parasite developmental cycle. For malaria vectors, the expression of anti-*Plasmodium* molecules should occur in the midgut or in the salivary glands: this aspect represents a critical step for an effective paratransgenic-based approach. When recombinant *Escherichia coli* was used for expressing anti-*Plasmodium berghei* in *An. stephensi*, the inhibition of the parasite was very limited. A possible explanation is the formation of insoluble inclusion bodies where the molecules were sequestered, preventing their secretion and diffusion (Riehle *et al.*, 2007). For this reason, the identification and optimization of strong secretion systems became pivotal for establishing recombinant bacterial strains for paratransgenic applications. The pioneering work on the mosquito symbiont *Pantoea agglomerans* offered an invaluable basis for progressing with bacterial genetic engineering for vector control (Bisi and Lampe, 2011). The repertoire

of anti-*Plasmodium* effector molecules consists of more than 30 molecules, classified in four categories on the basis of their mode of action: parasite killing; interaction with the parasite; interaction with the mosquito midgut and/or salivary glands epithelia; and manipulation of the mosquito immune system (Wang and Jacobs-Lorena, 2013 and references therein) (see Bottino-Rojas and James, Chapter 11; Guido *et al.*, Chapter 16, this volume). This divergence in pathogen inhibition mechanisms provides tools for developing synergistic anti-*Plasmodium* effector proteins, circumventing possible resistance phenomena by the parasite and maximizing its blockage.

In the past 5 years, a few studies have further substantiated *Asaia*'s potential for paratransgenic applications against mosquito-borne diseases. In this sense, the work proposed by D.J. Lampe and his collaborators is paradigmatic. Firstly, they recognized putative secreted proteins from *A. bogorensis* by a genetic screen using alkaline phosphatase gene fusions, identifying two promising candidates: a siderophore receptor protein and a YVTN beta-propeller repeat protein. The siderophore receptor gene was fused with genes encoding for anti-*Plasmodium* effectors, including the antimicrobial peptide Scorpine and the anti-Pbs21 scFv-Shiva1 immunotoxin. Once established in *An. stephensi*, paratransgenic *Asaia* significantly reduced *P. berghei* oocysts and the prevalence of mosquitoes carrying the parasites (Bongio and Lampe, 2015). This approach was further improved by the implementation of a conditional expression system: *Asaia* was engineered to produce anti-plasmodial toxins only after a bloodmeal. The engineered blood-inducible *Asaia* strains strongly inhibit parasite infection in *An. stephensi*, minimizing the effects on the fitness host, compared with the strains that constitutively express antiplasmodial molecules (Shane *et al.*, 2018). This strategy allowed the antiplasmodial bacterial strains to survive longer and be better transmitted through mosquito populations, creating an easily implemented and enduring vector control strategy.

More recently, another recombinant *Asaia* strain expressing *Wolbachia* surface protein (*wsp*) was designed. *Wolbachia* is the most common maternally transmitted endosymbiont of insects, currently in the forefront for population replacement and suppression strategies for mosquito control (Nazni *et al.*, 2019; Zheng *et al.*, 2019; Indriani *et al.*, 2020). *Wolbachia*, and more specifically *wsp*, is known to act as an inducer of the host innate immune system (Brattig *et al.*, 2004; Pinto *et al.*, 2012). Colonization with *wsp*-expressing *Asaia* was found to activate the immune response of *Ae. aegypti* and *An. stephensi* mosquitoes and inhibited the development of the heartworm parasite *Dirofilaria immitis* in *Ae. aegypti* by upregulating phagocytosis (Epis *et al.*, 2020). The same strain was also applied as an immuno-modulating agent able to stimulate macrophage response and induce a strong inhibition of *Leishmania* in *in vitro* assays (Varrotto-Boccazzi *et al.*, 2020).

15.4 *Asaia* in Mosquitoes: What Is its Beneficial Role?

Although *Asaia* tissue tropism and transmission routes in mosquitoes have been described, the identification of its contribution in host biology appears more challenging. *Asaia* beneficial function during developmental stages has been illustrated by B. Chouaia and collaborators (Chouaia *et al.*, 2012). They demonstrated that *Asaia* depletion by antibiotic treatment of breeding water of *An. stephensi* larvae caused a delay in development and an asynchronous progression into following stages, in parallel with an increase of their metabolic requirements (Chouaia *et al.*, 2012). Similarly, *Asaia* involvement in larval development was also described in *An. gambiae*, where the introduction of bacteria in the larval breeding sites caused a significant boost in developmental rate, impacting also on the expression of genes related to the formation of cuticle (Mitraka *et al.*, 2013). It is possible to speculate that a delay in larval development may expose mosquitoes to predators for longer periods, explaining the beneficial, although non-essential, contribution of *Asaia* symbiont to the host. Additionally, this

aspect could be beneficial and promising for mass rearing systems, in the perspective of using *Asaia* for vector control applications.

More recently, a study on *An. stephensi* using a highly specific anti-*Asaia* monoclonal antibody demonstrated the involvement of *Asaia* in the survival of adult males (Mancini *et al*., 2020). The description of the sex-specific phenotype after anti-*Asaia* mAb injection was correlated with transcriptional analysis of *Asaia*-carrying and *Asaia*-free individuals. The analysis of the differential gene expression suggests a broad and interconnected role of the symbiont in host physiology and survival, although specific phenomena have to be functionally investigated and the mechanism of action elucidated.

A comparative phylogenomic study on *Asaia* strains from different species of mosquitoes and from different populations of the Mediterranean fruit fly, *Ceratitis capitata*, revealed an interesting potential contribution of *Asaia* to the host. Most of the examined strains, in fact, harbour the pyrethroid hydrolase (PH) gene, which is likely to confer resistance to pyrethroids, the insecticides produced by plants and commonly used in pest treatments (Yadouleton *et al*., 2011; Comandatore *et al*., 2021). Although this involvement still needs to be directly demonstrated functionally, it potentially suggests substantial implications in the native and applicative role of this symbiont.

15.5 Considerations for Paratransgenic Applications of *Asaia*

Although insects hosting *Asaia* belong to a wide range of genera and originate in quite distant regions of the globe, cross-colonization experiments showed high transmission between bacterial strains and host species. *Asaia* strains isolated from *An. stephensi* were able to colonize other sugar-feeding and phylogenetically distant insect species like *Ae. aegypti* or the hemipthteran leafhopper *S. titanus* (Crotti *et al*., 2009). This evidence indicated that the introduction of modified *Asaia* into mosquito populations is

likely to bypass the genetic barriers of reproductively isolated taxa occurring in endemic malaria regions, which are known to hinder the success of vector control strategies (Wang and Jacobs-Lorena, 2013). If we refer only to malaria itself, out of 400 *Anopheles* mosquito species identified throughout the globe, only 40 species transmit human malaria. In Africa, the major vectors belong to complexes or groups of closely related species that may have highly variable behaviours and vectorial capacities. Within the *An. gambiae* complex, *An. gambiae sensu stricto* shows a significant chromosomal polymorphism related to ecological and behavioural adaptations (Lawniczak *et al*., 2010). A similar evolutionary phenomenon was observed in *An. funestus*, in which sympatric populations carrying specific chromosomal paracentric inversions showed restricted gene flow (Dia *et al*., 2011). Distribution of species from *An. nili* group and *An. moucheti* complexes, for example, are restricted to more humid regions of Africa; however, in some areas these species play the major role in malaria transmission (Antonio-Nkondjio *et al*., 2009). This ecological variety, together with the challenges of the intrinsic complexity of hosts genetic manipulation, favours the hypothesis of implementing paratransgenic approaches.

On the other hand, the great ability of *Asaia* to overcome inter-species boundaries may have implications on the spread of a transgene or genetically modified organism in the environment. This imposes the elaboration of an adequate risk assessment and regulatory programme designed to address and anticipate the real and perceived risks associated with the strategy before any field application (Aguilera *et al*., 2011). An urgent aspect to explore in field trials is the off-target effect on other organisms, likely cohabiting the target host or its ecological niche: these effects are unknown at the moment. Efforts are focused on the development of highly pathogen-specific effector molecules, in order to minimize any off-target effect.

These crucial safety aspects stress the concept that the releases of modified *Asaia* in the field (like any other genetically modified organism or symbiont) must be

approached with great caution and correlated with a thorough regulatory assessment. Moreover, public acceptance, community awareness and involvement represent other key features for control strategies. In order to develop and achieve success, campaigns of public and community engagement must represent a substantial and systematic part of field strategies development.

An additional aspect is the impact of bacteria on humans and their potential pathogenicity. A study specifically addressed these safety issues (Epis *et al.*, 2012a) by assessing *Asaia* spp. circulation among humans, in particular malaria patients and individuals exposed to repeated mosquito bites. No evidence of infections, even asymptomatic, caused by *Asaia* was observed. Other previous reports on *Asaia* infections in humans highlighted cases of *Asaia*-induced bacteraemia only in severely immuno-compromised patients (Tuuminen *et al.*, 2006; Juretschko *et al.*, 2010), suggesting that *Asaia* spp. bacteria should be classified as opportunistic pathogens for humans, with negligible pathogenicity in immuno-competent subjects.

15.6   Other Implications in *Asaia*–Host Interactions

Although paratransgenic approaches look like the most persuasive strategy, recent findings suggest that other *Asaia*-based approaches could be developed for tackling vector-borne diseases. Two simultaneous studies reported that *Asaia* colonization in different insect systems causes a modulation of the immune system of the host. Cappelli *et al.* (2019) showed that the supplement of serial doses of *Asaia* to *An. stephensi* induces the activation of the basal level of mosquito immunity and decreases the development of malaria parasites in the host. Similar findings were also reported in a study (Gonnella *et al.*, 2019) on leafhoppers, where *Asaia* was found to activate and up-regulate specific host immune genes. These findings expand the potential of harnessing *Asaia* for control of mosquito-borne diseases as a natural effector for mosquito immune

priming. On the other hand, other substantial implications were highlighted in a recent report where *A. bogorensis* proliferation in mosquito midgut after sugar uptake was associated with an increase in midgut pH and, in turn, with the enhancement of *Plasmodium* gametogenesis and greater permissiveness to the parasite infection (Wang *et al.*, 2021). Altogether, these studies indicate that *Asaia* is an important determinant in mosquito vector competence for *Plasmodium*, but further investigation of the regulation of the *Asaia*–*Plasmodium*–mosquito interactions is needed. In particular, given the partially conflicting aforementioned results, it would be interesting to verify whether the observed phenomena should be considered species-specific or even strain-specific.

15.7   Conclusions and Future Perspectives

Great interest has been devoted to novel approaches for mosquito control, due to the increasingly apparent operational limitations of traditional control methods (insecticides, source reduction, etc.). For this reason, in the past decade, a growing number of mosquito symbionts have been proposed as tools for paratransgenic applications and many studies aimed at verifying the potential of the most promising candidates against mosquito-borne diseases (Ricci *et al.*, 2012). *Asaia* exhibits a good potential for being harnessed for these purposes, due to its intrinsic features and its interactions with the mosquito host, as previously described and here summarized as follows.

1. Native *Asaia* was detected and isolated from *Anopheles* mosquitoes (the major malaria vectors), *Ae. aegypti* and *Ae. albopictus* (vectors of dengue and yellow fever) and from *Culex* spp. (vectors of West Nile virus). Therefore, it could be potentially employed in the control of several different mosquito-borne diseases.
2. Among many of the mosquito species/populations examined, *Asaia* was shown to be one of the predominant natural bacterial communities.

3. In mosquitoes, *Asaia* was found to naturally colonize aquatic developmental stages and, in adults, it localizes in the midgut, salivary glands and reproductive organs. This indicates the possibility of tackling parasite development in several tissues and to be vertically transmitted.

4. Although not directly demonstrated in the field, *Asaia* horizontal and, in particular, vertical transmission routes observed in the laboratory and in large cage set-ups suggest that it has the potential to ensure the rapid spread of modified strains within and between populations.

5. *Asaia* is cultivable in a cell-free medium and amenable to genetic transformation with exogenous DNA. There is evidence that recombinant strains of *Asaia* can rapidly infect and colonize recipient mosquitoes, colonizing their midgut, salivary glands and reproductive organs.

Although promising, the potential of *Asaia* for paratransgenesis is still based on laboratory evidence and assessed in small-scale studies. The *trait d'union* between the laboratory bench and the field is represented by the use of confined semi-field enclosures exposed to natural meteorological and lighting conditions. Population studies on *Asaia*-carrying mosquitoes will be pivotal for understanding its spreading and invasion potential, its competition with the natural microbiota and its effects on mosquito populations. Acquired data would be factored into a mathematical model as parameters for predicting the introduction, the performance dynamics and the impact of genetically modified *Asaia* in realistic field conditions.

In principle, the genetic manipulation of bacteria is simpler and faster than the genetic manipulation of mosquito vectors. Bacteria can be produced in large quantities and in many areas of the world and the logistics of introducing engineered bacteria into mosquito populations is easier than releasing genetically modified vectors. Another relevant aspect of the paratransgenic methodology is its complementarity with other control measures, in particular with traditional strategies (such as insecticide spray, insecticide-treated bednets).

The assessment of *Asaia* as an applicable paratransgenic tool is under way, since many aspects of its symbiotic association and its applicability still need to be fully elucidated. Nevertheless, the evidence supports a 'realistic optimism' toward the use of *Asaia*-based methodologies to integrate other control approaches against vector-borne diseases.

References

Aguilera, J., Gomes, A.R. and Nielsen, K.M. (2011) Genetically modified microbial symbiont as arthropod pest controllers: risk assessment through the European legislations. *Journal of Applied Entomology* 135, 494–502.

Akhoundi, M., Bakhtiari, R., Guillard, T., Baghaei, A., Tolouei, R. *et al.* (2012) Diversity of the bacterial and fungal microflora from the midgut and cuticle of phlebotomine sand flies collected in north-western Iran. *PLoS ONE* 7(11), e50259.

Aksoy, S., Weiss, B. and Attardo, G. (2008) Paratransgenesis applied for control of tsetse transmitted sleeping sickness. *Advances in Experimental Medicine and Biology* 627, 35–48.

Alonso, D.P., Mancini, M.V., Damiani, C., Cappelli, A., Ricci, I. *et al.* (2019). Genome reduction in the mosquito symbiont *Asaia*. *Genome Biology and Evolution* 11(1), 1–10.

Antonio-Nkondjio, C., Ndo, C., Costantini, C., Awono-Ambene, P., Fontenille, D. and Simard F. (2009) Distribution and larval habitat characterization of *Anopheles moucheti, Anopheles nili*, and other malaria vectors in river networks of southern Cameroon. *Acta Tropica* 112, 270–276.

Bae, S., Fleet, G.H. and Heard, G.M. (2006) Lactic acid bacteria associated with wine grapes from several Australian vineyards. *Journal of Applied Microbiology* 100, 712–727.

Barredo, E. and DeGennaro, M. (2020) Not just from blood: mosquito nutrient acquisition from nectar sources. *Trends in Parasitology* 36, 473–484.

Bextine, B., Lauzon, C., Potter, S., Lampe, D. and Miller, T.A. (2004) Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. *Current Microbiology* 48, 327–331.

Bilgo, E., Vantaux, A., Sanon, A., Ilboudo, S., Dabiré, R.K. *et al.* (2018) Field assessment of potential sugar feeding stations for disseminating bacteria in a paratransgenic approach to control malaria. *Malaria Journal* 17(1), 367.

Bisi, D.C. and Lampe, D.J. (2011) Secretion of anti-*Plasmodium* effector proteins from a natural *Pantoea agglomerans* isolate by using PelB and HlyA secretion signals. *Applied and Environmental Microbiology* 77, 4669–4675.

Bongio, N.J. and Lampe, D.J. (2015) Inhibition of *Plasmodium berghei* development in mosquitoes by effector proteins secreted from *Asaia* sp. bacteria using a novel native secretion signal. *PLoS ONE* 10(12), e0143541.

Brattig, N.W., Bazzocchi, C., Kirschning, C.J., Reiling, N., Buttner, D.W. *et al.* (2004) The major surface protein of *Wolbachia* endosymbionts in filarial nematodes elicits immune responses through TLR2 and TLR4. *The Journal of Immunology* 173, 437–445.

Cappelli, A., Damiani, C., Mancini, M.V., Valzano, M., Rossi, P. *et al.* (2019) *Asaia* activates immune genes in mosquito eliciting an anti-*Plasmodium* response: implications in malaria control. *Frontiers in Genetics* 10, 836.

Chouaia, B., Rossi, P., Montagna, M., Ricci, I., Crotti, E. *et al.* (2010) Molecular evidence for multiple infections as revealed by typing of *Asaia* bacterial symbionts of four mosquito species. *Applied and Environmental Microbiology* 76, 7444–7450.

Chouaia, B., Rossi, P., Epis, S., Mosca, M., Ricci, I. *et al.* (2012) Delayed larval development in *Anopheles* mosquitoes deprived of *Asaia* bacterial symbionts. *BMC Microbiology* 12 (Suppl. 1), S2.

Comandatore, F., Damiani, C., Cappelli, A., Ribolla, P.E.M., Gasperi, G. *et al.* (2021) Phylogenomics reveals that *Asaia* symbionts from insects underwent convergent genome reduction, preserving an insecticide-degrading gene. *mBio* 12(2), e00106-21.

Crotti, E., Damiani, C., Pajoro, M., Gonella, E., Rizzi, A. *et al.* (2009) *Asaia*, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders. *Environmental Microbiology* 11, 3252–3264.

Damiani, C., Ricci, I., Crotti, E., Rossi, P., Rizzi, A. *et al.* (2008) Paternal transmission of symbiotic bacteria in malaria vectors. *Current Biology* 18, 1087–1088.

Damiani, C., Ricci, I., Crotti, E., Rossi, P., Rizzi, A. *et al.* (2010) Mosquito–bacteria symbiosis: the case of *Anopheles gambiae* and *Asaia*. *Microbial Ecology* 60, 644–654.

De Freece, C., Damiani, C., Valzano, M., D'Amelio, S., Cappelli, A. *et al.* (2014) Detection and isolation of the α-proteobacterium *Asaia* in *Culex* mosquitoes. *Medical Veterinary Entomology* 28, 438–442.

De Vooght, L., Caljon, G., Stijlemans, B., De Baetselier, P., Coosemans, M. and Van den Abbeele, J. (2012) Expression and extracellular release of a functional anti-trypanosome Nanobody(R) in *Sodalis glossinidius*, a bacterial symbiont of the tsetse fly. *Microbial Cell Factories* 11, 23.

Dia, I., Sagnon, N., Guelbeogo, M.W. and Diallo, M. (2011) Bionomics of sympatric chromosomal forms of *Anopheles funestus* (Diptera: Culicidae). *Journal of Vector Ecology (Journal of the Society for Vector Ecology)* 36, 343–347.

Durvasula, R.V., Gumbs, A., Panackal, A., Kruglov, O., Aksoy, S. *et al.* (1997) Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. *Proceedings of the National Academy of Sciences USA* 94, 3274–3278.

Elston, K.M., Perreau, J., Maeda, G.P., Moran, N. and Barrick, J.E. (2021) Engineering a culturable *Serratia symbiotica* strain for aphid paratransgenesis. *Applied and Environmental Microbiology* 87(4), e02245-20.

Epis, S., Gaibani, P., Ulissi, U., Chouaia, B., Ricci, I. *et al.* (2012a) Do mosquito-associated bacteria of the genus *Asaia* circulate in humans? *European Journal of Clinical Microbiology & Infectious Diseases* 31, 1137–1140.

Epis, S., Montagna, M., Comandatore, F., Damiani, C., Diabaté, A. *et al.* (2012b) Molecular typing of bacteria of the genus *Asaia* in malaria vector *Anopheles arabiensis* Patton, 1905. *Journal of Entomological and Acarological Research* 44, e16.

Epis, S., Varotto-Boccazzi, I., Crotti, E., Damiani, C., Giovati, L. *et al.* (2020) Chimeric symbionts expressing a *Wolbachia* protein stimulate mosquito immunity and inhibit filarial parasite development. *Communication Biology* 3(1), 105.

Favia, G., Ricci, I., Damiani, C., Raddadi, N., Crotti, E. *et al.* (2007) Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. *Proceedings of the National Academy of Sciences USA* 104, 9047–9051.

Favia, G., Ricci, I., Marzorati, M., Negri, I., Alma, A. *et al.* (2008) Bacteria of the genus *Asaia*: a potential paratransgenic weapon against malaria. *Advances in Experimental Medicine and Biology* 627, 49–59.

Flórez, L., Scherlach, K., Gaube, P. *et al.* (2017) Antibiotic-producing symbionts dynamically transition between plant pathogenicity and insect-defensive mutualism. *Nature Communications* 8, 15172.

Gonella, E., Mandrioli, M., Tedeschi, R., Crotti, E., Pontini, M. and Alma, A. (2019) Activation of immune genes in leafhoppers by phytoplasmas and symbiotic bacteria. *Frontiers in Physiology* 10, 795.

Gottlieb, Y., Lalzar, I. and Klasson, L. (2015) Distinctive genome reduction rates revealed by genomic analyses of two *Coxiella*-like endosymbionts in ticks. *Genome Biology and Evolution* 7(6), 1779–1796.

Hurwitz, I., Fieck, A., Read, A., Hillesland, H., Klein, N. *et al.* (2011) Paratransgenic control of vector borne diseases. *International Journal of Biological Sciences* 7, 1334–1344.

Indriani, C., Tantowijoyo, W., Rancès, E., Andari, B., Prabowo, E. *et al.* (2020) Reduced dengue incidence following deployments of *Wolbachia*-infected *Aedes aegypti* in Yogyakarta, Indonesia: a quasi-experimental trial using controlled interrupted time series analysis. *Gates Open Research* 11(4), 50.

Juretschko, S., Beavers-May, T.K. and Stovall, S.H. (2010) Nosocomial infection with *Asaia lannensis* in two paediatric patients with idiopathic dilated cardiomyopathy. *Journal of Medical Microbiology* 59, 848–852.

Jacobs-Lorena, M. (2009) Using bacteria to contain the spread of malaria. In: *Progress and prospects for the use of genetically modified mosquitoes to inhibit disease transmission*. World Health Organization, Geneva, Switzerland, 4–6 May 2009.

Katsura, K., Kawasaki, H., Potacharoen, W., Saono, S., Seki, T. *et al.* (2001) *Asaia siamensis* sp. nov., an acetic acid bacterium in the alpha-proteobacteria. *International Journal of Systematic and Evolutionary Microbiology* 51, 559–563.

Lawniczak, M.K., Emrich, S.J., Holloway, A.K., Regier, A.P., Olson, M. *et al.* (2010) Widespread divergence between incipient *Anopheles gambiae* species revealed by whole genome sequences. *Science* 330, 512–514.

Li, F., Hua, H., Han, Y. and Hou, M. (2020). Plant-mediated horizontal transmission of *Asaia* between whitebacked planthoppers, *Sogatella furcifera*. *Frontiers in Microbiology* 11, 593485.

Mancini, M.V., Spaccapelo, R., Damiani, C., Accoti, A., Tallarita, M. *et al.* (2016) Paratransgenesis to control malaria vectors: a semi-field pilot study. *Parasite and Vectors* 10(9), 140.

Mancini, M.V., Damiani, C., Accoti, A., Tallarita, M., Nunzi, E. *et al.* (2018) Estimating bacteria diversity in different organs of nine species of mosquito by next generation sequencing. *BMC Microbiology* 18(1), 126.

Mancini, M.V., Damiani, C., Short, S.M., Cappelli, A., Ulissi, U., *et al.* (2020) Inhibition of *Asaia* in adult mosquitoes causes male-specific mortality and diverse transcriptome changes. *Pathogens* 9(5), 380.

Mangudo, C., Aparicio, J. and Gleiser, R. (2015) Tree holes as larval habitats for *Aedes aegypti* in urban, suburban and forest habitats in a dengue affected area. *Bulletin of Entomological Research* 105(6), 679–684.

Manzano-Marìn, A. and Latorre, A. (2016) Snapshots of a shrinking partner: genome reduction in *Serratia symbiotica*. *Scientific Reports* 6, 32590.

Marzorati, M., Alma, A., Sacchi, L., Pajoro, M., Palermo, S. *et al.* (2006) A novel *Bacteroidetes* symbiont is localized in *Scaphoideus titanus*, the insect vector of Flavescence doree in *Vitis vinifera*. *Applied and Environmental Microbiology* 72, 1467–1475.

Mitraka, E., Stathopoulos, S., Siden-Kiamos, I., Christophides, G.K. and Louis, C. (2013) *Asaia* accelerates larval development of *Anopheles gambiae*. *Pathogens and Global Health* 107(6), 305–311.

Müller, G.C., Beier, J.C., Traore, S.F., Toure, M.B., Traore, M.M. *et al.* (2010) Successful field trial of attractive toxic sugar bait (ATSB) plant-spraying methods against malaria vectors in the *Anopheles gambiae* complex in Mali, *West Africa. Malaria Journal* 21(9), 210.

Moore, J.E., McCalmont, M., Xu, J., Millar, B.C. and Heaney, N. (2002) *Asaia* sp., an unusual spoilage organism of fruit-flavored bottled water. *Applied and Environmental Microbiology* 68, 4130–4131.

Nazni, W.A., Hoffmann, A.A., Noor Afizah, A., Cheong, Y.L., Mancini, M.V. *et al.* (2019) Establishment of *Wolbachia* strain *w*AlbB in Malaysian populations of *Aedes aegypti* for dengue control. *Current Biology* 24, 4241–4248.

Pinto, S.B., Mariconti, M., Bazzocchi, C., Bandi, C. and Sinkins, S.P. (2012) *Wolbachia* surface protein induces innate immune responses in mosquito cells. *BMC Microbiology* 12 (Suppl. 1), S11.

Ricci, I., Damiani, C., Capone, A., De Freece, C., Rossi, P. and Favia, G. (2012) Mosquito/microbiota interactions: from complex relationships to biotechnological perspectives. *Current Opinion in Microbiology* 15, 278–284.

Riehle, M.A., Moreira, C.K., Lampe, D., Lauzon, C. and Jacobs-Lorena, M. (2007) Using bacteria to express and display anti-*Plasmodium* molecules in the mosquito midgut. *International Journal for Parasitology* 37, 595–603.

Robinson, C.J., Schloss, P., Ramos, Y., Raffa, K. and Handelsman, J. (2010) Robustness of the bacterial community in the cabbage white butterfly larval midgut. *Microbial Ecology* 59, 199–211.

Roux, O. and Robert, V. (2019) Larval predation in malaria vectors and its potential implication in malaria transmission: an overlooked ecosystem service? *Parasites and Vectors* 12, 217.

Ruiz, P., Sesena, S., Izquierdo, P.M. and Palop, M.L. (2010) Bacterial biodiversity and dynamics during malolactic fermentation of Tempranillo wines as determined by a culture-independent method. *Applied Environmental Microbiology* 86(5), 1555–1562

Shane, J.L., Grogan, C.L., Cwalina, C. and Lampe, D.J. (2018) Blood meal-induced inhibition of vector-borne disease by transgenic microbiota. *Nature Communications* 9(1), 4127.

Suzuki, R., Zhang, Y., Iino, T., Kosako, Y., Komagata, K. and Uchimura, T. (2010) *Asaia astilbes* sp. nov., *Asaia platycodi* sp. nov., and *Asaia prunellae* sp. nov., novel acetic acid bacteria isolated from flowers in Japan. *The Journal of General and Applied Microbiology* 56, 339–346.

Tang, M., Lv, L., Jing, S., Zhu, L. and He, G. (2010) Bacterial symbionts of the brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae). *Applied and Environmental Microbiology* 76, 1740–1745.

Taracena, M.L., Oliveira, P.L., Almendares, O., Umaña, C., Lowenberger, C. *et al.* (2015) Genetically modifying the insect gut microbiota to control Chagas disease vectors through systemic RNAi. *PLoS Neglected Tropical Diseases* 9, e0003358.

Taylor, L.H. (1999) Infection rates in, and the number of *Plasmodium falciparum* genotypes carried by *Anopheles* mosquitoes in Tanzania. *Annals of Tropical Medicine and Parasitology* 93, 659–662.

Tuuminen, T., Heinasmaki, T. and Kerttula, T. (2006) First report of bacteremia by *Asaia bogorensis*, in a patient with a history of intravenous-drug abuse. *Journal of Clinical Microbiology* 44, 3048–3050.

Varotto-Boccazzi, I., Epis, S., Arnoldi, I., Corbett, Y., Gabrieli, P. *et al.* (2020) Boosting immunity to treat parasitic infections: *Asaia* bacteria expressing a protein from *Wolbachia* determine M1 macrophage activation and killing of Leishmania protozoans. *Pharmacological Research* 161, 105288.

Wang, M., An, Y., Gao, L., Dong, S., Zhou, X. *et al.* (2021) Glucose-mediated proliferation of a gut commensal bacterium promotes *Plasmodium* infection by increasing mosquito midgut pH. *Cell Report* 35(3), 108992.

Wang, S. and Jacobs-Lorena, M. (2013) Genetic approaches to interfere with malaria transmission by vector mosquitoes. *Trends in Biotechnology* 109, 12734–12739.

Wang, S., Dos-Santo, A.L.A., Huang, W., Liu, K.C., Oshaghi, M.A. *et al.* (2017) Driving mosquito refractoriness to *Plasmodium falciparum* with engineered symbiotic bacteria. *Science* 357, 1399–1402.

Woruba, D.N., Morrow, J.L., Reynolds, O.L. *et al.* (2019) Diet and irradiation effects on the bacterial community composition and structure in the gut of domesticated teneral and mature Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae). *BMC Microbiology* 19, 281.

Yadouleton, A., Martin, T., Padonou, G., Chandre, F., Asidi, A. *et al.* (2011) Cotton pest management practices and the selection of pyrethroid resistance in *Anopheles gambiae* population in northern Benin. *Parasites & Vectors* 4, 60.

Yamada, Y., Katsura, K., Kawasaki, H., Widyastuti, Y., Saono, S. *et al.* (2000) *Asaia bogorensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the alpha-Proteobacteria. *International Journal of Systematic and Evolutionary Microbiology* 50, 823–829.

Yukphan, P., Potacharoen, W., Tanasupawat, S., Tanticharoen, M. and Yamada, Y. (2004) *Asaia krungthepensis* sp. nov., an acetic acid bacterium in the alpha-Proteobacteria. *International Journal of Systematic and Evolutionary Microbiology* 54, 313–316.

Zheng, X., Zhang, D., Li, Y. *et al.* (2019) Incompatible and sterile insect techniques combined eliminate mosquitoes. *Nature* 572, 56–61.

Zouache, K., Raharimalala, F.N., Raquin, V., Tran-Van, V., Raveloson, L.H. *et al.* (2011) Bacterial diversity of field-caught mosquitoes, *Aedes albopictus* and *Aedes aegypti*, from different geographic regions of Madagascar. *FEMS Microbiology Ecology* 75, 377–389.

16 Paratransgenesis in Mosquitoes and Other Insects: Microbial Ecology and Bacterial Genetic Considerations

Marisa L. Guido, Thomas P. Kelly, Nicholas J. Bongio and David J. Lampe*

Department of Biological Sciences, Duquesne University, Pittsburgh, Pennsylvania, USA

16.1 Introduction

The phenotype of any individual organism is the product of a complex interaction of the genotype and the environment. For insect vectors of plant and animal diseases, the phenotype of interest is the ability to transmit the pathogen in question. In principle, it should be possible to modify the vector phenotype to prevent disease transmission in two ways: (i) by modifying the genotype through transgenesis (i.e., adding or removing genes in the genome); or (ii) by modifying some aspect of the vector's internal environment. For vector insects, the environments in question are the tissues and structures necessary for pathogen replication and transmission. Very often these environments are co-colonized by symbiotic microorganisms. Manipulation of these microorganisms can lead to a change in the pathogen transmission phenotype of the insect. Changing the phenotype in this way is called paratransgenesis.

This review will cover efforts to implement paratransgenesis in mosquitoes and will focus mainly on efforts to control malaria. Several reviews have already covered related material (Coutinho-Abreu *et al.*, 2010; Wang and Jacobs-Lorena, 2013), therefore we focus mainly on the microbial ecology of mosquitoes relevant to paratransgenesis and bacterial genetic tools that may prove useful in creating paratransgenic bacterial strains suitable for release in the field. Although the examples used will emphasize mosquitoes, the methods should be generally applicable to paratransgenesis for other vectors of plant and animal diseases. Other paratransgenesis systems are covered in this volume (Weiss *et al.*, Chapter 13; Hurwitz *et al.*, Chapter 14; and Mancini and Favia, Chapter 15).

16.2 Requirements for Successful Paratransgenesis

Several requirements must be met for a paratransgenesis program to be successful.

1. Microbial ecology. Suitable microorganisms must be identified that live in the vector in proximity to the pathogen or parasite and that can be cultured in the laboratory. The microorganisms must be closely associated

---

*Corresponding author, email: lampe@duq.edu

with the vector in the life stage where transmission of the disease organisms occurs.

2. Effector molecules. Effectors must be discovered that inhibit the parasite or pathogen within the vector. The more specific the inhibition, the better, since unwanted and unpredicted interactions with non-target organisms can be reduced.

3. Effector delivery. The effectors must be delivered from the paratransgenesis organism in efficacious concentrations, at suitable periods of time, and in a location where they will interact with the disease-causing organism.

4. No fitness costs. Ideally, there should be no negative fitness effects on either the vector or the paratransgenic microorganisms.

5. Stable inheritance and other genetic considerations. The genes encoding the effectors must be inherited stably by the microorganisms without selection. The potential for horizontal transfer must be reduced or eliminated. No drug resistance genes should be released.

6. Introduction and spread. A means must be available to introduce and spread the paratransgenic strains in nature.

Each of these requirements will be discussed below. We would like to point out, however, that paratransgenesis can be considered a category of the emerging field of synthetic biology, which seeks to create new microorganisms with carefully defined phenotypes. Methods emerging from this field will no doubt find applications within paratransgenesis programmes, and the degree to which these methods can be standardized can only accelerate progress in paratransgenesis.

16.2.1 Mosquito microbial ecology

The microbial ecology of a wide range of insects has recently been reviewed (Guegan *et al*., 2018). The current understanding of the microbiota of mosquitoes has been recently reviewed as well (Guegan *et al*., 2018; Gao *et al*., 2020).

While the microbiota of mosquitoes has traditionally been investigated through culturable bacteria (Demaio *et al*., 1996;

Pumpuni *et al*., 1996; Straif *et al*., 1998; Gonzalez-Ceron *et al*., 2003; Pidiyar *et al*., 2004; Lindh *et al*., 2005; Rani *et al*., 2009; Cirimotich *et al*., 2011a; Dinparast Djadid *et al*., 2011; Joyce *et al*. 2011; Apte-Deshpande *et al*., 2012; Terenius *et al*., 2012; Valiente Moro *et al*., 2013; Ngwa *et al*., 2013; Arruda *et al*., 2021), 16S rRNA sequencing has led to more complete analyses of mosquito microbiomes (Guegan *et al*., 2018; Gao *et al*., 2020). The benefit of 16S sequencing primarily lies in the ability to identify bacterial species that are not easily cultured and offer a more complete look into the microbial makeup. The most important result from these studies, from a paratransgenesis standpoint, is that there does not appear to be any species of bacterium that forms an obligate association with mosquitoes, although many are quite common. In both *Anopheles* and *Aedes* mosquitoes, common genera include *Asaia*, *Acinetobacter*, *Aeromonas*, *Pantoea*, *Pseudomonas* and *Serratia* (Gendrin and Christophides, 2013; Wilke and Marrelli, 2015). *Comamonas*, *Elizabethkingia*, *Enterobacter* and *Klebsiella* are all commonly found in *Aedes* (Mancini *et al*., 2018), and *Escherichia–Shigella*, *Sphingomonas* and *Cupriavidus* are commonly found in *Anopheles* (Gendrin and Christophides, 2013) (see Mancini and Favia, Chapter 15, this volume, for more information on the genus *Asaia*). *Wolbachia* is commonly found in *Aedes* mosquitoes and may be a viable target for paratransgenic modification to combat dengue virus. In contrast, *Wolbachia* has been difficult to detect in *Anopheles* spp., although high-density infections were recently reported in *An. moucheti* and *An. memeilloni* (Walker *et al*., 2021). *Wolbachia* can be experimentally introduced into *Anopheles*, albeit with difficulty (Bian *et al*., 2013). It has been shown that the presence of *Asaia* within *Anopheles* mosquitoes impedes *Wolbachia* co-infection (Hughes *et al*., 2014; Rossi *et al*., 2015). For comprehensive tables listing the genera of bacteria found in numerous species of mosquitoes, see Minard *et al*. (2013) and Gao *et al*. (2020).

The role of the mosquito microbiota is not completely understood. Many presumed functions are reviewed in Minard *et al*.

(2013) and in Gao *et al.* (2020). Bacterial presence is important for larval development, and many anexic mosquitoes cannot develop past first-instar larvae (Coon *et al.*, 2014). The absence of microbes stunts larval development, but can be rescued by inoculation with a single bacterial species (Romoli *et al.*, 2021). Almost certainly, the microbiota is involved in nutrition, especially in adult females where it may contribute to the digestion of the bloodmeal (Gaio Ade *et al.*, 2011) and both in adult males and females where it may provide nutrients in addition to the sugar meals taken by both sexes (Minard *et al.*, 2013). In larvae, bacteria and other microorganisms provide a dietary source of fatty acids, especially linoleic acid, which is important later for adult development and physiology (Kominkova *et al.*, 2012). Gene expression differs in larvae lacking a microbiota, indicating that bacteria are responsible for some vitamin synthesis (Romoli *et al.*, 2021). It has been clearly demonstrated that the microbiota is involved in immune system activation in the midgut and that this activation plays a role in the ability of the mosquito to attack pathogens in the bloodmeal, especially *Plasmodium* spp. (Pumpuni *et al.*, 1996; Dong *et al.*, 2009; Cirimotich *et al.*, 2011b; Sharma *et al.*, 2013). Mosquitoes that have been 'primed' by exposure to bacteria are better at surviving bacterial infection later in life (Kulkarni *et al.*, 2021). Other roles for the microbiota include the ability to positively influence egg hatching and to aid in larval growth (Ponnusamy *et al.*, 2011; Chouaia *et al.*, 2012). Recently, it has been shown that bacterial symbionts have evolved to maintain genes associated with insecticide degradation (Comandatore *et al.*, 2021).

Exactly how mosquitoes acquire their microbiota is not entirely clear, although the microbiota changes dramatically depending on the life stage. Wang *et al.* (2011), for example, found that the microbiota of larvae and pupae of *Anopheles gambiae* was dominated by cyanobacteria, clearly a product of feeding in an aquatic environment, and the dominant bacteria changed significantly by adulthood. In some species, females deposit bacteria on the surface of eggs ('egg smearing')

and these can be acquired by larvae (Crotti *et al.*, 2009; Damiani *et al.*, 2010). During metamorphosis, the microbial community in the midgut is drastically altered and the gut is sterilized, or nearly so, via the formation of a meconium (Pumpuni *et al.*, 1996; Moll *et al.*, 2001), although there is evidence that some bacteria survive transstadially (Damiani *et al.*, 2008). The gut is repopulated in the adult stage either through bacteria acquired from the water from which the adults emerge or a plant nectar meal taken upon eclosion (Lindh *et al.*, 2005; Wang *et al.*, 2011; Minard *et al.*, 2013). Some species of bacteria, namely *Asaia* spp., can be passed from males to females during mating (Damiani *et al.*, 2008). The acquisition, composition and change of the mosquito microbiota remains an area where further study is needed. Studies in this area should be able to identify appropriate microbial species for paratransgenesis and inform strategies on how to spread paratransgenic strains in nature.

16.2.2   Effector molecules

After a suitable bacterial species is chosen for paratransgenesis, a critical step toward creating paratransgenic strains of bacteria is the isolation of antipathogen effectors. In principle, these can be any molecules that block pathogen development or transmission from the insect vector and can include proteins, peptides, small molecules, or RNAs. In this regard, the field of malaria paratransgenesis is rich with effectors and some of these may prove useful against other vector-borne diseases. Reviews of antimalarial effectors have been published (Caljon *et al.*, 2013; Wang and Jacobs-Lorena, 2013) (Bottino-Rojas and James, Chapter 11, this volume). Antiviral effectors (with an emphasis on transgenic insects) are covered by Franz (Chapter 22, this volume). We briefly treat each category below.

Proteins

Certain proteins have proved to be antimalarial. For example, a component of honey

bee venom, phospholipase A2, has demonstrated anti-malarial activity. Interestingly, its enzymatic activity is not necessary for its anti-malarial properties, since catalytically inactivated mutant versions of the protein are active against the parasite (Moreira *et al.*, 2002; Rodrigues *et al.*, 2008). There is speculation that the protein somehow alters the cell membranes of the mosquito midgut, thereby blocking parasite invasion of that tissue.

A very important class of anti-malarial proteins is antibodies and their derivatives (Kontermann, 2010). Several monoclonal antibodies have been isolated that bind to surface proteins of the reproductive stages of malaria parasites and block different parts of the mosquito life cycle (Rener *et al.*, 1983; Quakyi *et al.*, 1987; Barr *et al.*, 1991). Native antibodies are complex proteins consisting of four polypeptide chains linked by disulfide bonds. To use these antibodies in a paratransgenic strategy, the genes that encode the mature antibody must be converted into a single gene that encodes a protein that retains binding specificity and that can be translated and secreted by bacteria (Kipriyanov and Le Gall, 2004). Such constructs are called single-chain antibodies (scFvs) and are synthetic constructs that link the heavy-chain and light-chain variable regions of a specific antibody into a single open reading frame (ORF). When translated, this forms a protein that can reconstitute the specific binding structure of a mature vertebrate antibody. There are numerous variations on this basic scFv theme, including single-domain antibodies (containing only the heavy-chain binding region and sometimes called nanobodies), diabodies (constructs that bind to two different antigens) and scFv-toxins (constructs scFvs that are fused to a toxin gene) (Kontermann, 2010). The first successful demonstration of paratransgenesis against malaria used this latter kind of construct secreted by *Escherichia coli* in *Anopheles stephensi* to cause a decline in the number of oocysts formed in the midgut by *Plasmodium bergheii*, although the effect was weak (Yoshida *et al.*, 2001). There is extensive literature on the conversion of vertebrate IgGs to different single-chain forms (Fernandez, 2004).

Peptides

Peptides are another very important class of anti-malarial effectors, which may also be useful against many other pathogens. A major benefit of this class of effector is that they are typically short, sometimes no more than 20 amino acids in length. There are two important classes of effector peptides: peptides that directly kill the parasite and those that interfere with some specific parasite–vector interaction.

Peptides that kill pathogens or parasites have been derived from venoms, for example scorpine (from scorpion venom), or, more commonly, from peptides that are secreted as part of the eukaryotic innate immune system. Hundreds of these latter peptides have been described from many different eukaryotes, only a few of which have been used in paratransgenesis (Otvos, 2005; Nishie *et al.*, 2012; Vila-Farres *et al.*, 2012). This is, obviously, a promising area for future research. Indeed, the canonical paratransgenesis approach against Chagas disease transmitted by triatomine bugs used cecropin A (a peptide of the innate immune system of the giant silkmoth, *Hyalophora cecropia*) secreted by the Gram-positive bacterium *Rhodococcus rhodnii* to kill *Trypanasoma cruzi* (Hurwitz *et al.*, Chapter 14, this volume).

For peptides that do not directly interact with the parasite, considerations about the fitness cost for the carrier bacteria are recommended. For example, scorpine is a powerful antimicrobial and has the ability to kill both the parasite and paratransgenic bacteria that synthesize it. Drosomycin, while also an antimicrobial, is not effective against Gram-negative bacteria and could improve the fitness of the host bacteria (Tian *et al.*, 2008).

Other anti-malarial peptides interfere with specific interactions between the parasite and the mosquito host and block some important life stages from proceeding. For example, enolase–plasminogen interacting peptide (EPIP) is a six-amino acid peptide that blocks the interaction of plasminogen with ookinetes (Ghosh *et al.*, 2011). Since the ookinetes cannot invade the midgut wall, they die and the life cycle of the parasite is aborted. Another anti-malarial peptide of

this class is SM1 (salivary gland–midgut peptide 1), which blocks parasite–vector interactions in both the midgut and salivary glands of mosquitoes (Ghosh *et al.*, 2011). Midgut peptide 2, known as MP2, acts analogously to SM1, but functions against *Plasmodium falciparum*, whereas SM1 only has functionality against rodent malaria models (Vega-Rodriguez *et al.*, 2014).

Small molecules from biosynthetic pathways

Many small molecules are known that kill malaria parasites, ranging from drugs used to kill blood-stage parasites (e.g. artemisinin) to reactive oxygen species. Each of these molecules is the end product of some multigene biosynthetic pathway. In principle, it should be possible to move the pathway for these small molecules into a paratransgenesis species. For example, amorpha-4,11-diene, a precursor to the anti-malarial drug artemisinin, can be produced in *Escherichia coli* by adding heterologous genes from *Saccharomyces cerevisiae* and *Artemisia annua* (Tsuruta *et al.*, 2009).

RNAi

To our knowledge, small double-stranded RNAs (dsRNAs) have not been used as effector genes to interfere with parasite–vector interactions through RNA inhibition, although they could offer a powerful route to either kill parasites outright or block some important interaction with the vector. RNAi has been used in mosquitoes to knock out or repress specific genes by injecting the dsRNAs directly into adults. Delivery of dsRNA to the gut environment by bacteria expressing dsRNAs might target mosquito gut tissues or parasites directly. The RNase III protein from *Asaia* has been characterized to provide the groundwork for the use of RNAi paratransgenesis strategies (Asgari *et al.*, 2020). Delivery of dsRNA via bacteria to silence genes is a standard technique in *Caenorhabditis elegans* biology (Timmons and Fire, 1998). Strong caveats to using this method should be considered, especially the fact that many eukaryotic parasites lack a response to dsRNA because they lack the machinery to process it. For example,

Plasmodium spp. parasites and *Trypanosoma cruzi* lack this machinery, although *Trypanosoma brucei* retains it (Kolev *et al.*, 2011). Obviously, the potential effectiveness of RNAi would have to be evaluated on an individual pathogen/vector basis. Other aspects of RNAi are covered by De Schutter and Smagghe (Chapter 4, this volume).

The need to express more than one effector

Paratransgenic strains of bacteria that express a single effector against a parasite or pathogen may lose effectiveness quickly. This is because the effector provides strong selective pressure and favours those parasite or pathogen variants that are resistant to the effector. The scientific literature is replete with instances of the failure of a drug or resistance strategy when only a single effector or drug is used. Bacteria, viruses, eukaryotic pathogens, agricultural insect pests, weedy plants and even cancer cells all display this phenomenon as the simple and entirely predictable result of directional natural selection. Therefore, any paratransgenic strain that will be released into nature should express more than one effector. Importantly, the effectors should act in different ways to minimize the chances that resistant parasites or pathogens will evolve. A simple way to do this would be to construct an artificial operon with two or more different effector genes driven by the same promoter. Another strategy to express multiple effectors is engineering them as a polyprotein. This would help eliminate issues with differential expression of the different effectors. This was attempted by Wang *et al.* (2017) when they created a construct that produced five different effectors fused together as a polyprotein. Interestingly, the strains expressing this construct performed no better than those expressing scorpine alone, suggesting that there is room for improvement in how these polyproteins are constructed.

16.2.3   Effector delivery

Once effectors are discovered that kill or impair the pathogen of interest, a way to

deliver these from the paratransgenic bacterial species must be found. If the paratransgenesis bacterium is Gram-positive, efficient secretion of proteins or peptides can be achieved using simple secretion signals added to the N-terminus of the effector constructs. This was the method used to secrete cecropin A from *Rhodococcus rhodnii* in the Chagas disease paratransgenesis system (Durvasula *et al.*, 1997).

Effector delivery is more complicated if the paratransgenesis species is Gram-negative. Gram-negative bacteria have two cell membranes bounding a compartment known as the periplasm that separates the cytoplasm from the exterior of the cell. Any protein or peptide that is to be secreted has to traverse this complicated barrier. Passage through the periplasm is beneficial in some cases, however, as it allows certain proteins to fold properly and form disulfide bonds within a reducing environment. In nature, Gram-negative bacteria have evolved at least nine different mechanisms to secrete proteins and some of these have proved useful to secrete foreign proteins (Holland, 2010). A comprehensive series of reviews of protein secretion by bacteria has recently been published (Sandkvist *et al.*, 2019). Two of these mechanisms and the structure of the Gram-negative cell membranes and periplasm are shown in Fig. 16.1.

Heterologous secretion systems

As a first step toward achieving secretion of effectors from a new paratransgenesis species, heterologous systems can be employed. Two effective ones are the haemolysin A (HlyA) autotransporter secretion system from pathogenic *E. coli* and the *pelB* leader sequence from the pectate lyase gene of *Erwinia carotovora*.

Haemolysin A is a toxin encoded by the *hlyA* gene in the haemolysin operon of particular pathogenic strains of *E. coli* (Blight and Holland, 1994; Holland, 2010). This secretion system is designated as Type I, or an autotransporter. These sorts of secretion systems are generally dedicated to the secretion of a single protein and usually accomplish secretion in one energy-dependent step across both the inner and outer membranes of the Gram-negative cell. Studies of various deletion constructs of HlyA have demonstrated that a C-terminal 46–50 amino acid sequence directs the protein toward the secretion apparatus (Kenny *et al.*, 1992). That apparatus consists of the HlyB and HlyD proteins encoded by the haemolysin operon in addition to TolC, a native outer membrane protein. Together, HlyB, HlyD and TolC form the transport mechanism that moves HlyA from the cytoplasm to the outside of the cell. Any protein or peptide linked to the HlyA signal sequence can also be transported using this highly efficient system (Tzschaschel *et al.*, 1996; Fernandez *et al.*, 2000; Bisi and Lampe, 2011). Using the HlyA system, Wang *et al.* (2012) were able to secrete seven different anti-malarial effector proteins from the Gram-negative bacterial species *Pantoea agglomerans* in both *Anopheles gambiae* and *An. stephensi* midguts.

Two caveats should be mentioned when considering HlyA. The first is that, although very efficient, proteins secreted in this way do not spend any time in the periplasmic space (Holland, 2010). The periplasm is an important environment that allows many proteins to fold correctly before final secretion takes place. Secondly, the HlyA system depends on the presence of a host membrane protein, TolC. The success of malaria paratransgenesis in *P. agglomerans* using HlyA was achieved in no small measure due to the fact that *P. agglomerans* has a native TolC protein that can function with the *E. coli* HlyA system (Bisi and Lampe, 2011; Wang *et al.*, 2012).

Another heterologous system that has proved effective at secreting foreign proteins is the *pelB* secretion system. PelB encodes the pectate lysase gene which is secreted from the plant pathogen *E. carotovora* (Lei *et al.*, 1987). The PelB protein is secreted via a Type II secretory mechanism, a category that includes several different pathways grouped together under what is termed the general secretory pathway (Sec) (Fig. 16.1). This pathway is universal in Gram-negative bacteria and seems to be homologous to secretion from eukaryotic cells (see reviews of

(A) Haemolysin secretion   **(B)** Type II secretion
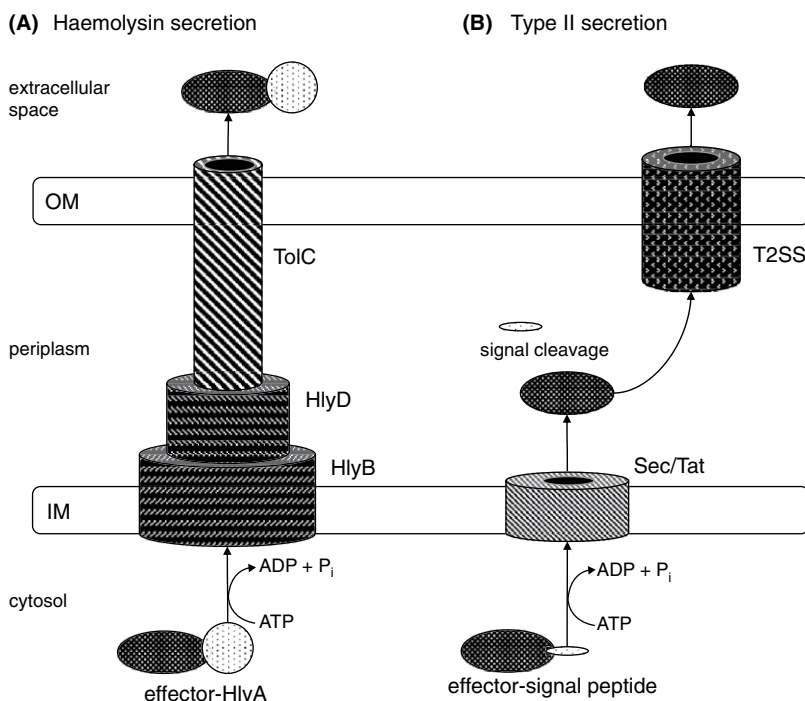
Fig. 16.1. Secretion of effector proteins by bacteria using one of two different pathways. (A) The haemolysin autotransporter system. **(B)** The type II general secretory pathway. Both systems are highly simplified in this figure. Effector proteins destined for secretion carry either a ca. 50-amino acid C-terminal signal sequence for the HlyA pathway or a short N-terminal signal sequence for the Type II secretion pathway. The signals differ in Type II depending on whether or not they will cross the inner membrane via either the twin-arginine pathway (Tat) or the general secretory pathway (Sec). The haemolysin system consists of HlyB, HlyD and a native TolC protein that assemble to create a pore that spans the inner and outer membranes. Proteins using this pathway are secreted directly into the extracellular space in one energy-dependent step. Effectors targeted to the Type II secretion system cross the inner membrane and spend time in the periplasm where the signal sequence is cleaved and they may undergo disulfide bond formation. These proteins cross the outer membrane in many ways that are not completely understood. For a fuller discussion see Korotkov *et al*. (2012) and Holland *et al*. (2016).

the system in Pugsley *et al.*, 2004; Holland, 2010). Proteins that are synthesized with short N-terminal hydrophobic leader sequences are targeted to the inner membrane, where they pass through the membrane in an energy-dependent process involving numerous proteins in the secretion apparatus. Once in the periplasm, proteins cross the outer membrane to be inserted into the outer membrane or to be secreted outside the cell. Protein passage out of the cell is mediated by numerous mechanisms, many of which are poorly understood. Type II secretion using leader sequences like *pelB* have been very successful in secretion of a variety of proteins, especially scFvs and their variants (Dammeyer *et al.*, 2011). One clarification of this system should be made. The use of the *pelB* leader is extremely efficient in targeting proteins to the periplasm. Their escape from the cell, however, may not be through *bona fide* secretion, and may be due to non-specific release due to bacterial lysis or release of outer membrane vesicles. The same is true for other Type II signal sequences used in this way (Grogan *et al.*, 2021).

There are many positive features of using heterologous Type II secretion signal sequences. The main one is simplicity. The addition of a *pelB* or other signal sequence to

a gene encoding an effector protein is trivial, unlike the HlyA system which requires the presence of several other heterologous proteins. The downside to using Type II secretion is that, while simple, proteins cannot be targeted easily to exit past the outer membrane. Thus, proteins secreted by Type II secretion often get 'stuck' in the periplasm, which can have deleterious effects on cell fitness. We and others have noticed a strong correlation between efficient secretion and cell fitness (Bisi and Lampe, 2011).

Native secretion systems

If heterologous secretion systems fail to function, it may be necessary to develop native secretion systems for a given bacterial species. If a genome sequence is available, it can be mined using bioinformatic methods to identify candidate secretion signals (Nielsen *et al.*, 1997; Petersen *et al.*, 2011). Bioinformatic methods are likely to miss many secreted proteins, since it is known that many secreted proteins contain leaders that do not conform well or at all to any secretion consensus sequence (Payne *et al.*, 2012), although these methods are being constantly improved. A recently published comprehensive platform for identifying substrates secreted by various Gram-negative secretion pathways is called BastionHub (Wang *et al.*, 2021). This method takes advantage of experimentally verified substrates secreted by known pathways to predict secreted proteins and is a material improvement over previous methods.

16.2.4 Fitness considerations for paratransgenic bacteria

As noted at the beginning of this chapter, the fitness of paratransgenic organisms must be kept in mind. Ultimately, paratransgenic strains destined for release into the environment must compete with wild bacteria. Any severe fitness disadvantage will eliminate them before they can colonize vectors and suppress parasites or pathogens. Areas where fitness concerns can be addressed include ensuring efficient secretion (see above) and the use of conditional promoters (see below). Ultimately, fitness levels of paratransgenic strains will have to be measured empirically. When comparing paratransgenic strains to wild strains that may be encountered upon release, measurements to consider include comparisons of maximum growth rate, relative growth in competitive co-culture and the relative ability to colonize the targeted vector (Shane *et al.*, 2018).

Transcription of effector genes

Effector genes must be transcribed in the paratransgenesis species. Ideally, transcription would be strong, ensuring an abundance of the effector product. The kind of transcription necessary for a particular paratransgenesis programme should be considered carefully. Does the effector need to be produced continuously or only under the specific conditions when the pathogen or parasite is present? Can the two conditions be distinguished? The answers to these questions will determine what kind of promoter to use to drive effector gene transcription.

Constitutive promoters

If a continuous supply of effector gene product is desired, the gene should be placed under the control of a constitutive promoter. Heterologous constitutive promoters exist that function in a wide variety of bacterial species. One such promoter is that from the neomycin phosphotransferase II gene (*nptII*) (Auerswald *et al.*, 1981; Beck *et al.*, 1982; Reiss *et al.*, 1984). If a genome sequence is available, then the native promoters for genes like the homologues of *E. coli rpsL* (30S ribosomal subunit protein S12) and *groEL* (an *E. coli* chaperonin) should be considered. Of course, any gene expressed in a constitutive manner can be considered as long as the promoter is sufficiently active to produce the desired amount of effector gene transcript. For the laboratory demonstration of paratransgenesis for malaria using *P. agglomerans* and some *Asaia bogorensis*

strains, we used the *nptII* promoter (Wang *et al.*, 2012; Bongio and Lampe, 2015; Grogan *et al.*, 2021).

Conditional promoters

If the parasite or pathogen enters the insect vector under specific conditions, then the use of conditional promoters should be considered. Female mosquitoes that transmit malaria, for example, acquire the parasite in a bloodmeal, which leads to a dramatically different gut environment than does sugar feeding. Conditional promoters offer distinct advantages over constitutive ones in this context. In particular, bacterial strains that only express the antipathogen effector proteins under narrow conditions are more likely to be able to compete with wild strains, since the potential fitness cost of expressing the effectors is limited in time.

Bacterial genetic screens have been developed to detect genes that are activated under specific conditions. The most common and successful of these screens are *in vivo* expression technology (IVET) and differential fluorescence induction (DFI). Many permutations of both of these screens have been reviewed, as well as their relative strengths and weaknesses (Rediers *et al.*, 2005; Jackson and Giddens, 2006; Hsiao and Zhu, 2009). Conditional promoters can also be discovered using next-generation transcriptomic techniques (Hor *et al.*, 2018; Perez-Sepulveda and Hinton, 2018; Kozinska *et al.*, 2019).

Finally, conditional promoters may be uncovered by identifying homologues of genes known to be activated by certain conditions in other bacterial species. For example, bloodmeal-induced conditional promoters were identified in the mosquito symbiont *Asaia bogorensis* by comparison with blood-activated genes from other bacteria. These were utilized for antiplasmodial paratransgenesis (Shane *et al.*, 2018). As expected, strains conditionally expressing the antiplasmodial effector scorpine demonstrated significantly increased fitness when compared with a constitutive strain.

16.2.5 Genetically stable paratransgenic strains suitable for field release

As noted earlier, paratransgenic strains suitable for field release should be genetically stable, should not carry introduced drug resistance genes and should be resistant to horizontal transfer. Chromosomal insertion by a variety of means and suitably constructed plasmids can meet these demands. In other cases, special plasmids can be employed that differ significantly from the plasmids used for routine laboratory use.

Insertion of effector constructs into the chromosome

An alternative to using transposons that insert into many sites in the genome is to use one that has only a single insertion site, namely Tn7. An extensive review of the biology of Tn7 can be found in Craig *et al.*, 2002 (and see O'Brochta, Chapter 1, this volume). Tn7 inserts into *attTn7*, or the Tn7 attachment site, which is a sequence found downstream of the highly conserved *glmS* gene encoding glutamine synthetase. Interestingly, the *attTn7* site is present in many bacteria, so it may have wide applicability (Craig, 1996). Insertion of Tn7 into *attTn7* causes few, if any, fitness effects and so is very attractive in terms of creating paratransgenic strains (Craig *et al.*, 2002). A large number of broad host-range constructs have been developed for Tn7 (Choi *et al.*, 2005; Choi and Schweizer, 2006). The main drawback in using Tn7 is, of course, that *attTn7* must be present for it to function.

Alternatives to site-specific transposition involve the use of transposons like *Himar1* which insert essentially randomly throughout the chromosome (e.g., Rubin *et al.*, 1999). Obviously, there may be fitness costs to *Himar1* insertions and so these would need to be evaluated on an insertion-by-insertion basis. Vectors for insertion of *Himar1* into essentially any bacterial species have been developed (Fig. 16.2).

The second general method to introduce effectors into the chromosome is recombination. The most common kind of recombination occurs when a single crossover event
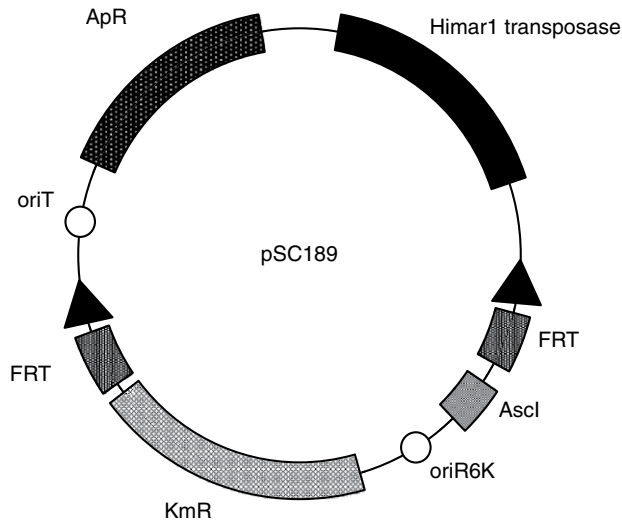
Fig. 16.2. pSC189, a broad host-range transposon insertion vector based on *Himar1*. pSC189 is used to make random insertions into the chromosome of many bacterial species. See text and Chiang and Rubin (2002) for details. ApR, ampicillin resistance; KmR, kanamycin resistance; *oriT*, origin of transfer; *Himar1* transposase, transposase gene; FRT, recombination sites of yeast FLP recombinase; *Asc*I, insertion site for exogenous DNA; oriR6K, a conditional origin of replication. Solid triangles indicated the transposon inverted terminal repeat sequences.

occurs between a plasmid carrying the effector construct and a homologous site on the chromosome. This results in the integration of the entire plasmid into the chromosome and such events are common enough that they are easily produced and isolated.

Ideally, only the effector gene and its promoter and terminator would be recombined into the chromosome. This requires a double crossover event, which is much rarer. Such events can be increased in frequency by orders of magnitude by supplying enzymes that mediate recombination of foreign DNA into the chromosome. The most highly developed of these is the Red recombination system derived from bacteriophage lambda (Datta *et al.*, 2006; Thomason *et al.*, 2007). The frequency of Red recombination is so high in *E. coli* that no drug marker is necessary to recover chromosomal recombination events (Sawitzke *et al.*, 2007). It is likely that this technique will work in many Gram-negative bacteria and the recombination machinery for Red recombination has been placed on broad host-range plasmids for just this use (Datta *et al.*, 2006).

In strains where Red recombination is difficult to implement, alternate two-step recombination methods may be implemented. For example, the inclusion of counter-selectable markers such as the *Bacillus subtilis sacB* gene on the integration plasmid can be used to increase recovery of sequential crossover events (Reyrat *et al.*, 1998). When expressed in Gram-negative bacteria, *sacB* confers sucrose toxicity. As such, growing transformants in the presence of sucrose allows for isolation of transformants that have undergone sequential crossover events via elimination of those that retain a copy of *sacB* in their chromosome. Recombination events can also be stimulated via double-strand breaks in the chromosome. Nucleases with large recognition sites unlikely to be found in most genomes, such as the I-*Sce*I endonuclease from *S. cerevisiae*, are used for this purpose (Posfai *et al.*, 1999). Assuming the genome of the target strain does not include this recognition site, it can be included on the integration plasmid used for recombination. The nuclease can then be conditionally expressed after the

first crossover event, resulting in a double-strand break within the initial site of recombination. When such breaks occur, bacterial double-strand break repair mechanisms kick in, most often resulting in homologous recombination at the site of the break. This in turn results in greater recovery of transformants having undergone a subsequent crossover event. As counter-selection and stimulated recombination are not mutually exclusive, they can also be combined for greater efficiency (Cianfanelli *et al*., 2020).

Finally, CRISPR/Cas is a relatively new genetic manipulation technique consisting of DNA-encoded RNA-mediated sequence-specific targeting of DNA (Concha and Papa, Chapter 7, this volume). Initially developed from the adaptive immune system of *Streptococcus pyogenes*, the main components of CRISPR/Cas genome editing systems are RNA-guided Cas endonucleases and a single-guide RNA (sgRNA) that is specific to the desired DNA target (Jinek *et al*., 2012). If a protospacer adjacent motif (PAM) sequence is present, the sgRNA guides the Cas endonuclease to the target DNA sequence, which is subsequently cleaved in a manner dependent on the type of Cas system being used. This technique is attractive in that chromosomal manipulation can be achieved without selectable marker genes. This makes CRISPR/Cas systems significantly faster, as removal of a marker is unnecessary. However, CRISPR/Cas systems can be toxic to some bacterial strains with inefficient DNA repair mechanisms (Liu *et al*., 2020). Different bacterial strains may also be limited in number of editable sites, given the necessity that specific PAM motifs are present near all targeted DNA.

Strains with plasmids

Insertion of effector genes into the chromosome may not yield enough product to affect pathogen development significantly. A solution to this problem is the use of plasmids, which often exist as multi-copy episomes. Use of plasmids in this way for effector delivery faces two major problems for deployment in the field related to biosafety: (i) the common use of drug-resistance genes to maintain plasmids in the cells that carry them; and (ii) the propensity of plasmids to undergo horizontal transfer to other species of bacteria. Fortunately, both of these problems can be overcome using bacterial genetics. A good model system for 'biosafe' plasmids suitable for field use is the GeneGuard system developed by Wright *et al*. (2015) which is described briefly in Fig. 16.3. GeneGuard plasmids use conditional origins of replication, broad host-range toxin–antidote systems and auxotrophic complementation to ensure biosafety (Wright *et al*., 2015).

Conditional origins of replication (COR)

Natural plasmids undergo autonomous replication using a replication protein (rep) that binds to a *cis*-acting origin of replication on the plasmid itself. Normally rep and its origin are on the same plasmid, ensuring autonomous replication. Removing the rep gene from the plasmid and inserting it into the bacterial chromosome makes the plasmid conditional in the sense that the plasmid can only replicate in that strain (or similar strains) of bacteria where rep is supplied in *trans*. A common configuration of this sort is the oriR6K/pir gene system commonly used in *E. coli*. If such a plasmid were horizontally transferred, it could not replicate unless the rep protein is present, which is extremely unlikely.

Another advantage to CORs is the fact that plasmid copy number can be altered by altering the expression or sequence of the Rep protein. Kittleson *et al*. (2011) altered the ribosomal binding site of repA and pir and screened for copy number variants using a strategy known as DIfferent ALleles of rep (DIAL). They were able to isolate strains that produced plasmids that varied in copy number over two orders of magnitude simply by altering the amount of Rep protein that was translated by the cell, introducing not only biosafety, but copy number variation as well (Kittleson *et al*., 2011).
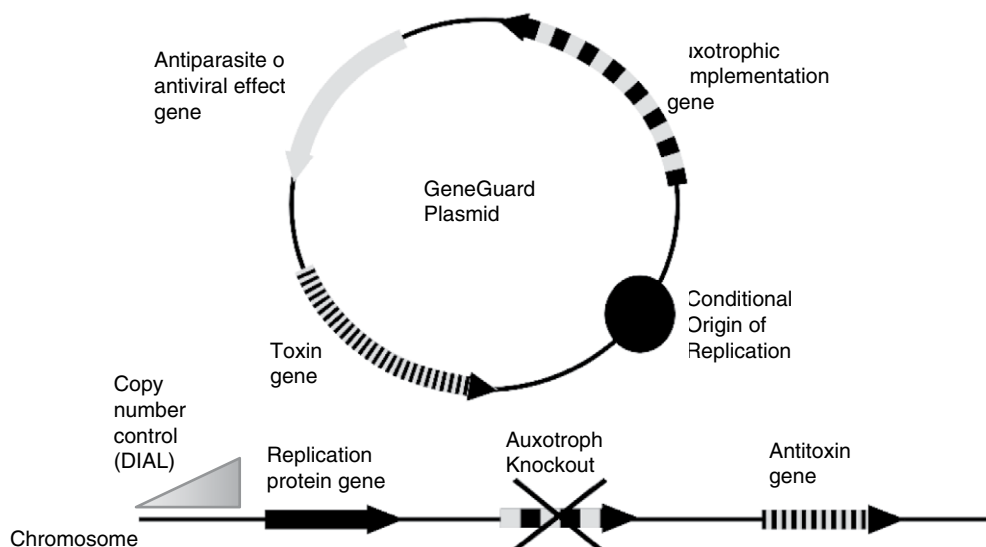
Fig. 16.3. Biosafe plasmids for use in paratransgenesis. A plasmid based on GeneGuard (Wright *et al*., 2015) principles for paratransgenesis that can be maintained without drug selection and that is resistant to horizontal transfer is shown. The origin of replication is dependent on a gene inserted into the chromosome and the copy number can be varied by varying the ribosome binding site (DIAL). The plasmid is maintained because it complements an auxotrophic gene knocked out in the chromosome. Horizontal transfer is suppressed with the toxin carried by the plasmid. It is complemented by an antitoxin gene in the chromosome. Finally, the plasmid carries some kind of anti-parasite or antiviral effector.

Toxin–antidote systems

Conditional origins of replication are inherently biosafe since the rep gene is separated from the origin of replication on the plasmids. Biosafety can be enhanced by including toxin–antitoxin systems. Such systems occur naturally on plasmids to promote plasmid stability in the cells that acquire them (Burga *et al*., 2020). In that context, a gene encoding a long-lived toxin co-occurs on the plasmid with another gene encoding a short-lived antitoxin. If the plasmid is lost from the cell, the antitoxin decays before the toxin and the cell dies, thus ensuring plasmid retention. The toxin and antitoxin genes can be separated to prevent horizontal transfer, which is done in the GeneGuard platform. For GeneGuard, the antitoxin is placed on the chromosome while the toxin is carried on the plasmid. If the plasmid were to be horizontally transferred, the toxin would kill the recipient cell. Since the antitoxin

is in the host cell, however, the plasmid is maintained and the cell can survive.

Auxotrophic complementation

Laboratory plasmids are usually maintained by selection for drug resistance, which is convenient and efficient, but not ethically suitable for release into the field. Firstly, intentional release of drug resistance is unsafe. Secondly, there is no way to select for drug resistance in the field, so the plasmids can be lost. Auxotrophic complementation is a solution to this problem. Here, a gene essential for growth is knocked out in the host strain. A gene complementing this loss can be provided in *trans* on a plasmid, automatically selecting for the retention of the plasmid. In the case of Geneguard, dapA (encoding dihydrodipicolinate synthase) was used. In principle, any gene required for growth in the host could be employed for this approach.

16.2.6 Introducing and spreading bacterial strains for paratransgenesis

A critical step in the success of a paratransgenesis programme is the introduction and spread of paratransgenic microorganisms within a vector population in nature. Bacterial species that form an obligate association with vectors are ideal in this context, since, once obtained by the vector, they will spread naturally via the biology of the vector itself. As noted above, our current knowledge of mosquito microbiota suggests that such obligate associations do not exist, although some species are quite common. Many common species of bacteria that live in adult mosquitoes, like *Pantoea* spp. and *Asaia* spp., are also found in floral nectar, suggesting that mosquitoes acquire these members of the microbiota from sugar meals.

The use of bacterial strains that do not have inherent drive mechanisms such as that of *Wolbachia* or that do not form obligate associations with their vectors will require a careful choice of bacterial species and clever uses of vector behaviour. *Asaia* (reviewed by Mancini and Favia, Chapter 15, this volume) is a genus that has very attractive microbial ecology. These species infect mosquito midguts, salivary glands and ovaries, multiply with the bloodmeal, are spread from males to females during copulation, and are deposited on eggs by the female (Favia *et al*., 2007; Damiani *et al*., 2008; Crotti *et al*., 2009). These properties suggest that paratransgenic *Asaia* strains might be easily spread in the field.

Spreading bacteria to mosquitoes in the field will also require taking advantage of mosquito behaviour. One suggestion has been to take advantage of the fact that the females of many mosquito species require sugar meals in addition to blood, while males exclusively sugar feed. It may be possible to spread paratransgenic strains by sugar meals supplemented with strong attractants (Muller *et al*., 2010; Beier *et al*., 2012).

Finally, the concern exists that paratransgenesis may be irreversible. While it is necessary for paratransgenic strains to persist for long enough to have their desired antipathogen effect, it may also be desirable that these strains be subject to some kind of control. Similar concerns have been expressed about gene drive systems in transgenic insects, which has led to the development of self-limiting drive systems (Raban and Akbari, Chapter 8; Champer, Chapter 9, this volume). Similar ideas exist for self-limiting paratransgenesis. Self-limiting paratransgenesis is the idea that, especially with strains using plasmids, the plasmids would be lost if not selected for. This idea was tested using standard laboratory plasmids in *Serratia marcesans* and it was found that, without selections, the plasmids persisted for about 130 bacterial generations and three mosquito generations before transgenic bacteria could no longer be detected (Huang *et al*., 2020). GeneGuard plasmids are expected to behave similarly (Wright *et al*., 2015).

16.3 Paratransgenesis of Mosquitoes Against Malaria with Genetically Modified Bacteria

Using the strategies outlined above, we successfully demonstrated paratransgenesis against malaria in the laboratory using transgenic strains of *P. agglomerans* (Wang *et al*., 2012). This species was isolated from a laboratory colony of *Anopheles stephensi* and was later selected to persist in the midgut of these flies for up to 14 days (Riehle *et al*., 2007). *P. agglomerans* is a gamma-proteobacterium in the order Enterobacterales, the same order to which *E. coli* belongs. Strains expressing various anti-malarial peptides and proteins were able to reduce the prevalence of females carrying any oocysts from 90% to 18% after an infectious bloodmeal (Wang *et al*., 2012). A reduction in prevalence was measured in both *An. gambiae* carrying *P. falciparum* and in *An. stephensi* carrying *P. berghei*, suggesting that these strains may work in any *Anopheles* vector species against multiple different species of *Plasmodium*. This is an important consideration and a distinct advantage of paratransgenesis against malaria, since there are five species

of *Plasmodium* that cause malaria in humans transmitted efficiently by at least 40 species of *Anopheles* (Sinka *et al.*, 2012).

Another bacterial species, *Asaia* sp. strain SF2.1, was engineered to conditionally express the antiplasmodial effector scorpine within the mosquito midgut. Four native bloodmeal-inducible promoters were identified, verified and amplified within plasmids to conditionally express scorpine in the presence of blood. These plasmids were transformed into *Asaia*, which was fed to *An. stephensi* in a sugar meal prior to a *P. berghei*-infected bloodmeal. Three of four strains conditionally expressing scorpine significantly reduced prevalence of infection compared with both the negative control and the constitutive positive control (Shane *et al.*, 2018). Other strains of this species carrying other paratransgenic constructs have also been constructed (Bongio and Lampe, 2015; Grogan *et al.*, 2021).

Finally, a strain of *S. marcesens*, AS1, was engineered to produce and secrete a number of antiplasmodial effector proteins. The AS1 strain, isolated from *Anopheles* ovaries, stably colonizes the mosquito midgut and is transmitted through mosquito populations horizontally and vertically. Strains of AS1 were engineered to secrete the anti-*Plasmodium* effectors Shiva1, (EPIP)4, (MP2)2, mPLA2, scorpine or a fusion protein consisting of all five using the HasA exporting system. *An. gambiae* were fed strains in sugar meals prior to a *P. falciparum*-infected bloodmeal. All strains significantly inhibited oocyst development compared with the control (Wang *et al.*, 2017).

16.4 Paratransgenesis with Naturally Occurring Bacterial Strains

So far we have described strategies to genetically engineer bacteria with favourable microbial ecology into paratransgenesis strains. Interestingly, some bacterial strains are naturally paratransgenic. For example, Cirimotich *et al.* (2011a) isolated 16 culturable bacterial species from the midguts of *An. gambiae* collected in Zambia. One of these strains belonged to the genus *Enterobacter* as determined by 16S sequencing. This strain, Esp_Z, was capable of completely inhibiting *P. falciparum* development in the midgut of *An. gambiae*. Additional experiments showed that the factor responsible for the inhibition was diffusible, heat insensitive and most likely some kind of reactive oxygen species, since the effect could be eliminated through the presence of the antioxidant, vitamin C. One hypothesis to account for the presence of this strain in *An. gambiae* is that it was selected for because it offers some kind of advantage to the mosquitoes that carry it by reducing the load of *P. falciparum* in the mosquitoes. If this hypothesis were correct, we might expect to see Esp_Z widespread in African *An. gambiae*, but it is not. An alternative hypothesis is that the anti-*Plasmodium* properties of Esp_Z are fortuitous and were selected for some other reason, most likely competition with other bacteria. If this hypothesis is correct, many other naturally occurring bacterial strains will prove to be antiplasmodial. Strains like these have the advantage of not having been genetically engineered; however, they may lack the appropriate microbial ecology and their discovery requires the mass screening of culturable bacteria from wild mosquitoes.

16.5 Conclusions

Reducing the ability of insects to transmit pathogens through paratransgenesis offers a promising route to decrease the burden of many of the most important human diseases, such as malaria and dengue fever. Although the creation of paratransgenic strains has many facets, the genetic tools are available to modify nearly any bacterial species. These include broad host-range plasmids, constitutive promoters, broad host-range transposons, an abundance of effectors, and simple genetic screens to isolate secretion signals and conditional promoters.

Many challenges remain, especially gaining an improved understanding of insect microbial ecology and methods to

deliver and spread paratransgenic species in nature. The use of metagenomics in different mosquito species will begin to clarify the nature of the mosquito microbiome and suggest bacterial species for which specialized culture techniques can be developed so that these species can be developed as paratransgenesis platforms. An increasing understanding of the microbial ecology of mosquitoes will also suggest ways to deliver paratransgenic bacterial species in the field to ensure their spread throughout a vector population. Finally, a systems biology approach to paratransgenesis and a standardization of vector construction will aid in the rapid development of paratransgenic strains for many mosquito species, as well as other important insect vectors.

References

Apte-Deshpande, A., Paingankar, M., Gokhale, M.D. and Deobagkar, D.N. (2012) *Serratia odorifera* a midgut inhabitant of *Aedes aegypti* mosquito enhances its susceptibility to dengue-2 virus. *PLoS ONE* 7, e40401.

Arruda, A., Ferreira, G.E.M., Santos Jr, A., Matos, N.B., Carvalho, T.S. *et al.* (2021) Diversity of culturable bacteria isolated from the feces of wild *Anopheles darlingi* (Diptera: Culicidae) mosquitoes from the Brazilian Amazon. *Journal of Medical Entomology* 58, 1900–1907.

Asgari, M., Ilbeigikhamsehnejad, M., Rismani, E., Dinparast Djadid, N. and Raz, A. (2020) Molecular characterization of RNase III protein of Asaia sp. for developing a robust RNAi-based paratransgensis tool to affect the sexual life-cycle of *Plasmodium* or *Anopheles* fitness. *Parasites & Vectors* 13, 42.

Auerswald, E.A., Ludwig, G. and Schaller, H. (1981) Structural analysis of Tn5. *Cold Spring Harbor Symposia on Quantitative Biology* 45 (Pt 1), 107–113.

Barr, P.J., Green, K.M., Gibson, H.L., Bathurst, I.C., Quakyi, I.A. and Kaslow, D.C. (1991) Recombinant Pfs25 protein of *Plasmodium falciparum* elicits malaria transmission-blocking immunity in experimental animals. *Journal of Experimental Medicine* 174, 1203–1208.

Beck, E., Ludwig, G., Auerswald, E.A., Reiss, B. and Schaller, H. (1982) Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. *Gene* 19, 327–336.

Beier, J.C., Muller, G.C., Gu, W., Arheart, K.L. and Schlein, Y. (2012) Attractive toxic sugar bait (ATSB) methods decimate populations of *Anopheles malaria* vectors in arid environments regardless of the local availability of favoured sugar-source blossoms. *Malaria Journal* 11**,** 31.

Bian, G., Joshi, D., Dong, Y., Lu, P., Zhou, G. *et al.* (2013) *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to *Plasmodium* infection. *Science* 340, 748–751.

Bisi, D.C. and Lampe, D.J. (2011) Secretion of anti-*Plasmodium* effector proteins from a natural *Pantoea agglomerans* isolate by using PelB and HlyA secretion signals. *Applied and Environmental Microbiology* 77, 4669–4675.

Blight, M.A. and Holland, I.B. (1994) Heterologous protein secretion and the versatile *Escherichia coli* haemolysin translocator. *Trends in Biotechnology* 12, 450–455.

Bongio, N.J. and Lampe, D.J. (2015) Inhibition of *Plasmodium berghei* development in mosquitoes by effector proteins secreted from *Asaia* sp. bacteria using a novel native secretion signal. *PLoS ONE* 10, e0143541.

Burga, A., Ben-David, E. and Kruglyak, L. (2020) Toxin–antidote elements across the Tree of Life. *Annual Review of Genetics* 54, 387–415.

Caljon, G., De Vooght, L. and Van Den Abbeele, J. (2013) Options for the delivery of anti-pathogen molecules in arthropod vectors. *Journal of Invertebrate Pathology* 112 (Suppl.), S75–82.

Chiang, S.L. and Rubin, E.J. (2002) Construction of a mariner-based transposon for epitope-tagging and genomic targeting. *Gene* 296, 179–185.

Choi, K.H. and Schweizer, H.P. (2006) mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. *Nature Protocols* 1, 153–161.

Choi, K.H., Gaynor, J.B., White, K.G., Lopez, C., Bosio, C.M. *et al.* (2005) A Tn7-based broad-range bacterial cloning and expression system. *Nature Methods* 2, 443–448.

Chouaia, B., Rossi, P., Epis, S., Mosca, M., Ricci, I. *et al.* (2012) Delayed larval development in *Anopheles* mosquitoes deprived of *Asaia* bacterial symbionts. *BMC Microbiology* 12 (Suppl. 1), S2.

Cianfanelli, F.R., Cunrath, O. and Bumann, D. (2020) Efficient dual-negative selection for bacterial genome editing. *BMC Microbiology* 20, 129.

Cirimotich, C.M., Dong, Y., Clayton, A.M., Sandiford, S.L., Souza-Neto *et al.* (2011a) Natural microbe-mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*. *Science* 332, 855–858.

Cirimotich, C.M., Ramirez, J.L. and Dimopoulos, G. (2011b) Native microbiota shape insect vector competence for human pathogens. *Cell Host & Microbe* 10, 307–310.

Comandatore, F., Damiani, C., Cappelli, A., Ribolla, P.E.M., Gasperi, G. *et al.* (2021) Phylogenomics reveals that *Asaia* symbionts from insects underwent convergent genome reduction, preserving an insecticide-degrading gene. *mBio* 12, e00106-21.

Coon, K.L., Vogel, K.J., Brown, M.R. and Strand, M.R. (2014) Mosquitoes rely on their gut microbiota for development. *Molecular Ecology* 23, 2727–2739.

Coutinho-Abreu, I.V., Zhu, K.Y. and Ramalho-Ortigao, M. (2010) Transgenesis and paratransgenesis to control insect-borne diseases: current status and future challenges. *Parasitology International* 59, 1–8.

Craig, N.L. (1996) Transposon Tn7. *Current Topics in Microbiology and Immunology* 204, 27–48.

Craig, N.L., Craigie, R. Gellert, M. and Lambowitz, A. (eds) (2002) *Mobile DNA II*. American Society for Microbiology Press, Washington, DC.

Crotti, E., Damiani, C., Pajoro, M., Gonella, E., Rizzi, A. *et al.* (2009) *Asaia*, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders. *Environmental Microbiology* 11, 3252–3264.

Damiani, C., Ricci, I., Crotti, E., Rossi, P., Rizzi, A. *et al.* (2010) Mosquito-bacteria symbiosis: the case of *Anopheles gambiae* and *Asaia*. *Microbial Ecology* 60**,** 644–654.

Damiani, C., Ricci, I., Crotti, E., Rossi, P., Rizzi, A., Scuppa, P., Esposito, F., Bandi, C., Daffonchio, D. and Favia, G. (2008) Paternal transmission of symbiotic bacteria in malaria vectors. *Current Biology* 18, R1087–1088.

Dammeyer, T., Steinwand, M., Kruger, S.C., Dubel, S., Hust, M. and Timmis, K.N. (2011) Efficient production of soluble recombinant single chain Fv fragments by a *Pseudomonas putida* strain KT2440 cell factory. *Microbial Cell Factories* 10, 11.

Datta, S., Costantino, N. and Court, D.L. (2006) A set of recombineering plasmids for Gram-negative bacteria. *Gene* 379, 109–115.

Demaio, J., Pumpuni, C.B., Kent, M. and Beier, J.C. (1996) The midgut bacterial flora of wild *Aedes triseriatus*, *Culex pipiens*, and *Psorophora columbiae* mosquitoes. *American Journal of Tropical Medicine and Hygiene* 54, 219–223.

Dinparast Djadid, N., Jazayeri, H., Raz, A., Favia, G., Ricci, I. and Zakeri, S. (2011) Identification of the midgut microbiota of *An. stephensi* and *An. maculipennis* for their application as a paratransgenic tool against malaria. *PLoS ONE* 6, e28484.

Dong, Y., Manfredini, F. and Dimopoulos, G. (2009) Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathogens* 5, e1000423.

Durvasula, R.V., Gumbs, A., Panackal, A., Kruglov, O., Aksoy, S. *et al.* (1997) Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. *Proceedings of the National Academy of Sciences USA* 94, 3274–3278.

Favia, G., Ricci, I., Damiani, C., Raddadi, N., Crotti, E. *et al.* (2007) Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. *Proceedings of the National Academy of Sciences USA* 104, 9047–9051.

Fernandez, L.A. (2004) Prokaryotic expression of antibodies and affibodies. *Current Opinion in Biotechnology* 15, 364–373.

Fernandez, L.A., Sola, I., Enjuanes, L. and De Lorenzo, V. (2000) Specific secretion of active single-chain Fv antibodies into the supernatants of *Escherichia coli* cultures by use of the hemolysin system. *Applied and Environmental Microbiology* 66, 5024–5029.

Gaio Ade, O., Gusmao, D.S., Santos, A.V., Berbert-Molina, M.A., Pimenta, P.F. and Lemos, F.J. (2011) Contribution of midgut bacteria to blood digestion and egg production in *Aedes aegypti* (Diptera: Culicidae) (L.). *Parasites and Vectors* 4, 105.

Gao, H., Cui, C., Wang, L., Jacobs-Lorena, M. and Wang, S. (2020) Mosquito microbiota and implications for disease control. *Trends in Parasitology* 36, 98–111.

Gendrin, M. and Christophides, G.K. (2013) The *Anopheles* mosquito microbiota and their impact on pathogen transmission. In*:* Manguin, S. (ed.) *Anopheles Mosquitoes – New Insights into Malaria Vectors*. IntechOpen, London, pp. 525–548. Available at: https://www.intechopen.com/books/anopheles-mosquitoes-new-insights-into-malaria-vectors/the-anopheles-mosquito-microbiota-and-their-impact-on-pathogen-transmission (accessed 14 April 2022)

Ghosh, A.K., Coppens, I., Gardsvoll, H., Ploug, M. and Jacobs-Lorena, M. (2011) *Plasmodium* ookinetes coopt mammalian plasminogen to invade the mosquito midgut. *Proceedings of the National Academy of Sciences USA* 108, 17153–17158.

Gonzalez-Ceron, L., Santillan, F., Rodriguez, M.H., Mendez, D. and Hernandez-Avila, J.E. (2003) Bacteria in midguts of field-collected *Anopheles albimanus* block *Plasmodium vivax* sporogonic development. *Journal of Medical Entomology* 40, 371–374.

Grogan, C., Bennett, M., Moore, S. and Lampe, D. (2021) Novel *Asaia bogorensis* signal sequences for *Plasmodium* inhibition in *Anopheles stephensi*. *Frontiers in Microbiology* 12, 633667.

Guegan, M., Zouache, K., Demichel, C., Minard, G., Tran Van, V. *et al.* (2018) The mosquito holobiont: fresh insight into mosquito-microbiota interactions. *Microbiome* 6, 49.

Holland, I.B. (2010) The extraordinary diversity of bacterial protein secretion mechanisms. *Methods in Molecular Biology* 619, 1–20.

Holland, I.B., Peherstorfer, S., Kanonenberg, K., Lenders, M., Reimann, S. and Schmitt, L. (2016) Type I protein secretion – deceptively simple yet with a wide range of mechanistic variability across the family. *EcoSal Plus* 7, 1–46.

Hor, J., Gorski, S.A. and Vogel, J. (2018) Bacterial RNA biology on a genome scale. *Molecular Cell* 70, 785–799.

Hsiao, A. and Zhu, J. (2009) Genetic tools to study gene expression during bacterial pathogen infection. *Advances in Applied Microbiology* 67, 297–314.

Huang, W., Wang, S. and Jacobs-Lorena, M. (2020) Self-limiting paratransgenesis. *PLoS Neglected Tropical Diseases* 14, e0008542.

Hughes, G.L., Dodson, B.L., Johnson, R.M., Murdock, C.C., Tsujimoto, H. *et al.* (2014) Native microbiome impedes vertical transmission of *Wolbachia* in *Anopheles* mosquitoes. *Proceedings of the National Academy of Sciences USA* 111, 12498–12503.

Jackson, R.W. and Giddens, S.R. (2006) Development and application of in vivo expression technology (IVET) for analysing microbial gene expression in complex environments. *Infectious Disorders – Drug Targets* 6, 207–240.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821.

Joyce, J.D., Nogueira, J.R., Bales, A.A., Pittman, K.E. and Anderson, J.R. (2011) Interactions between La Crosse virus and bacteria isolated from the digestive tract of *Aedes albopictus* (Diptera: Culicidae). *Journal of Medical Entomology* 48, 389–394.

Kenny, B., Taylor, S. and Holland, I.B. (1992) Identification of individual amino acids required for secretion within the haemolysin (HlyA) C-terminal targeting region. *Molecular Microbiology* 6, 1477–1489.

Kipriyanov, S.M. and Le Gall, F. (2004) Generation and production of engineered antibodies. *Molecular Biotechnology* 26, 39–60.

Kittleson, J.T., Cheung, S. and Anderson, J.C. (2011) Rapid optimization of gene dosage in *E. coli* using DIAL strains. *Journal of Biological Engineering* 5, 10.

Kolev, N.G., Tschudi, C. and Ullu, E. (2011) RNA interference in protozoan parasites: achievements and challenges. *Eukaryotic Cell* 10, 1156–1163.

Kominkova, D., Rejmankova, E., Grieco, J. and Achee, N. (2012) Fatty acids in anopheline mosquito larvae and their habitats. *Journal of Vector Ecology* 37, 382–395.

Kontermann, R.E. (2010) Alternative antibody formats. *Current Opinion in Molecular Therapeutics* 12, 176–183.

Korotkov, K.V., Sandkvist, M. and Hol, W.G. (2012) The type II secretion system: biogenesis, molecular architecture and mechanism. *Nature Reviews Microbiology* 10**,** 336-351.

Kozinska, A., Seweryn, P. and Sitkiewicz, I. (2019) A crash course in sequencing for a microbiologist. *Journal of Applied Genetics* 60, 103–111.

Kulkarni, A., Pandey, A., Trainor, P., Carlisle, S., Chhilar, J.S. *et al.* (2021) Trained immunity in *Anopheles gambiae*: antibacterial immunity is enhanced by priming via sugar meal supplemented with a single gut symbiotic bacterial strain. *Frontiers in Microbiology* 12, 649213.

Lei, S.P., Lin, H.C., Wang, S.S., Callaway, J. and Wilcox, G. (1987) Characterization of the *Erwinia carotovora* pelB gene and its product pectate lyase. *Journal of Bacteriology* 169, 4379–4383.

Lindh, J.M., Terenius, O. and Faye, I. (2005) 16S rRNA gene-based identification of midgut bacteria from field-caught *Anopheles gambiae sensu lato* and *A. funestus* mosquitoes reveals new species related to known insect symbionts. *Applied and Environmental Microbiology* 71, 7217–7223.

Liu, Z., Dong, H., Cui, Y., Cong, L. and Zhang, D. (2020) Application of different types of CRISPR/Cas-based systems in bacteria. *Microbial Cell Factories* 19, 172.

Mancini, M.V., Damiani, C., Accoti, A., Tallarita, M., Nunzi, E. *et al.* (2018) Estimating bacteria diversity in different organs of nine species of mosquito by next generation sequencing. *BMC Microbiology* 18, 126.

Minard, G., Tran, F.H., Raharimalala, F.N., Hellard, E., Ravelonandro, P. *et al.* (2013) Prevalence, genomic and metabolic profiles of *Acinetobacter* and *Asaia* associated with field-caught *Aedes albopictus* from Madagascar. *FEMS Microbiology Ecology* 83, 63–73.

Moll, R.M., Romoser, W.S., Modrzakowski, M.C., Moncayo, A.C. and Lerdthusnee, K. (2001) Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. *Journal of Medical Entomology* 38, 29–32.

Moreira, L.A., Ito, J., Ghosh, A., Devenport, M., Zieler, H. *et al.* (2002) Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes. *Journal of Biological Chemistry* 277, 40839–40843.

Muller, G.C., Beier, J.C., Traore, S.F., Toure, M.B., Traore, M.M. *et al.* (2010) Successful field trial of attractive toxic sugar bait (ATSB) plant-spraying methods against malaria vectors in the *Anopheles gambiae* complex in Mali, *West Africa. Malaria Journal* 9, 210.

Ngwa, C.J., Glockner, V., Abdelmohsen, U.R., Scheuermayer, M., Fischer, R. *et al.* (2013) 16S rRNA gene-based identification of *Elizabethkingia meningoseptica* (Flavobacteriales: Flavobacteriaceae) as a dominant midgut bacterium of the Asian malaria vector *Anopheles stephensi* (Dipteria: Culicidae) with antimicrobial activities. *Journal of Medical Entomology* 50, 404–414.

Nielsen, H., Engelbrecht, J., Brunak, S. and Von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering* 10, 1–6.

Nishie, M., Nagao, J. and Sonomoto, K. (2012) Antibacterial peptides "bacteriocins": an overview of their diverse characteristics and applications. *Biocontrol Science* 17, 1–16.

Otvos, L. Jr (2005) Antibacterial peptides and proteins with multiple cellular targets. *Journal of Peptide Science* 11, 697–706.

Payne, S.H., Bonissone, S., Wu, S., Brown, R.N., Ivankov, D.N. *et al.* (2012) Unexpected diversity of signal peptides in prokaryotes. *mBio* 3, e00339-12

Perez-Sepulveda, B.M. and Hinton, J.C.D. (2018) Functional transcriptomics for bacterial gene detectives. *Microbiology Spectrum* 6(5).

Petersen, T.N., Brunak, S., Von Heijne, G. and Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods* 8, 785–786.

Pidiyar, V.J., Jangid, K., Patole, M.S. and Shouche, Y.S. (2004) Studies on cultured and uncultured microbiota of wild *Culex quinquefasciatus* mosquito midgut based on 16s ribosomal RNA gene analysis. *American Journal of Tropical Medicine and Hygiene* 70, 597–603.

Ponnusamy, L., Boroczky, K., Wesson, D.M., Schal, C. and Apperson, C.S. (2011) Bacteria stimulate hatching of yellow fever mosquito eggs. *PLoS ONE* 6, e24409.

Posfai, G., Kolisnychenko, V., Bereczki, Z. and Blattner, F.R. (1999) Markerless gene replacement in *Escherichia coli* stimulated by a double-strand break in the chromosome. *Nucleic Acids Research* 27, 4409–4415.

Pugsley, A.P., Francetic, O., Driessen, A.J. and De Lorenzo, V. (2004) Getting out: protein traffic in prokaryotes. *Molecular Microbiology* 52, 3–11.

Pumpuni, C.B., Demaio, J., Kent, M., Davis, J.R. and Beier, J.C. (1996) Bacterial population dynamics in three anopheline species: the impact on *Plasmodium* sporogonic development. *American Journal of Tropical Medicine and Hygiene* 54, 214–218.

Quakyi, I.A., Carter, R., Rener, J., Kumar, N., Good, M.F. and Miller, L.H. (1987) The 230-kDa gamete sur-face protein of *Plasmodium falciparum* is also a target for transmission-blocking antibodies. *Journal of Immunology* 139, 4213–4217.

Rani, A., Sharma, A., Rajagopal, R., Adak, T. and Bhatnagar, R.K. (2009) Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected *Anopheles stephensi* – an Asian malarial vector. *BMC Microbiology* 9, 96.

Rediers, H., Rainey, P.B., Vanderleyden, J. and De Mot, R. (2005) Unraveling the secret lives of bacteria: use of in vivo expression technology and differential fluorescence induction promoter traps as tools for exploring niche-specific gene expression. *Microbiology and Molecular Biology Reviews* 69, 217–261.

Reiss, B., Sprengel, R. and Schaller, H. (1984) Protein fusions with the kanamycin resistance gene from transposon Tn5. *EMBO Journal* 3, 3317–3322.

Rener, J., Graves, P.M., Carter, R., Williams, J.L. and Burkot, T.R. (1983) Target antigens of transmis-sion-blocking immunity on gametes of plasmodium falciparum. *Journal of Experimental Medicine* 158, 976–981.

Reyrat, J.M., Pelicic, V., Gicquel, B. and Rappuoli, R. (1998) Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. *Infection and Immunity* 66, 4011-4017.

Riehle, M.A., Moreira, C.K., Lampe, D., Lauzon, C. and Jacobs-Lorena, M. (2007) Using bacteria to express and display anti-*Plasmodium* molecules in the mosquito midgut. *International Journal for Parasitology* 37, 595–603.

Rodrigues, F.G., Santos, M.N., De Carvalho, T.X., Rocha, B.C., Riehle, M.A. *et al.* (2008) Expression of a mutated phospholipase A2 in transgenic *Aedes fluviatilis* mosquitoes impacts *Plasmodium gallinace-um* development. *Insect Molecular Biology* 17, 175–183.

Romoli, O., Schonbeck, J.C., Hapfelmeier, S. and Gendrin, M. (2021) Production of germ-free mosquitoes via transient colonisation allows stage-specific investigation of host-microbiota interactions. *Nature Communications* 12, 942.

Rossi, P., Ricci, I., Cappelli, A., Damiani, C., Ulissi, U. *et al.* (2015) Mutual exclusion of *Asaia* and *Wolbachia* in the reproductive organs of mosquito vectors. *Parasites & Vectors* 8, 278.

Rubin E.J., Akerley B.J., Novik V.N., Lampe D.J., Husson R.N. and Mekalanos J.J. (1999) In vivo transpos-ition of mariner-based elements in enteric bacteria and mycobacteria. *Proceedings of the National Academy of Sciences USA* 96, 1645.

Sandkvist, M., Cascales, E. and Christie, P.J. (2019) *Protein Secretion in Bacteria*. ASM Press, Wash-ington, DC.

Sawitzke, J.A., Thomason, L.C., Costantino, N., Bubunenko, M., Datta, S. and Court, D.L. (2007) Recombi-neering: in vivo genetic engineering in *E. coli*, *S. enterica*, and beyond. *Methods in Enzymology* 421, 171–199.

Shane, J.L., Grogan, C.L., Cwalina, C. and Lampe, D.J. (2018) Blood meal-induced inhibition of vec-tor-borne disease by transgenic microbiota. *Nature Communications* 9, 4127.

Sharma, A., Dhayal, D., Singh, O.P., Adak, T. and Bhatnagar, R.K. (2013) Gut microbes influence fitness and malaria transmission potential of Asian malaria vector *Anopheles stephensi*. *Acta Tropica* 128, 41–47.

Sinka, M.E., Bangs, M.J., Manguin, S., Rubio-Palis, Y., Chareonviriyaphap, T. *et al.* (2012) A global map of dominant malaria vectors. *Parasites & Vectors* 5, 69.

Straif, S.C., Mbogo, C.N., Toure, A.M., Walker, E.D., Kaufman, M. *et al.* (1998) Midgut bacteria in *Anopheles gambiae* and *An. funestus* (Diptera: Culicidae) from Kenya and Mali. *Journal of Medical Entomology* 35, 222–226.

Terenius, O., Lindh, J.M., Eriksson-Gonzales, K., Bussiere, L., Laugen, A.T. *et al.* (2012) Midgut bacterial dynamics in *Aedes aegypti*. *FEMS Microbiology Ecology* 80, 556–565.

Thomason, L., Court, D.L., Bubunenko, M., Costantino, N., Wilson, H. *et al.* (2007) Recombineering: gen-etic engineering in bacteria using homologous recombination. *Current Protocols in Molecular Biology* 106, 1.16.1–1.16.39.

Tian, C., Gao, B., Rodriguez, M. del C., Lanz-Mendoza, H., Ma, B. and Zhu, S. (2008) Gene expression, antiparasitic activity, and functional evolution of the drosomycin family. *Molecular Immunology* 45, 3909–3916.

Timmons, L. and Fire, A. (1998) Specific interference by ingested dsRNA. *Nature* 395, 854.

Tsuruta, H., Paddon, C.J., Eng, D., Lenihan, J.R., Horning, T. *et al.* (2009) High-level production of amor-pha-4,11-diene, a precursor of the antimalarial agent artemisinin, in *Escherichia coli*. *PLoS ONE* 4, e4489.

Tzschaschel, B.D., Guzman, C.A., Timmis, K.N. and De Lorenzo, V. (1996) An *Escherichia coli* hemolysin transport system-based vector for the export of polypeptides: export of Shiga-like toxin IIeB subunit by *Salmonella typhimurium* aroA. *Nature Biotechnology* 14, 765–769.

Valiente Moro, C., Tran, F.H., Raharimalala, F.N., Ravelonandro, P. and Mavingui, P. (2013) Diversity of culturable bacteria including Pantoea in wild mosquito *Aedes albopictus*. *BMC Microbiology* 13, 70.

Vega-Rodriguez, J., Ghosh, A.K., Kanzok, S.M., Dinglasan, R.R., Wang, S. *et al.* (2014) Multiple pathways for *Plasmodium* ookinete invasion of the mosquito midgut. *Proceedings of the National Academy of Sciences USA* 111, E492–500.

Vila-Farres, X., Giralt, E. and Vila, J. (2012) Update of peptides with antibacterial activity. *Current Medicinal Chemistry* 19, 6188–6198.

Walker, T., Quek, S., Jeffries, C.L., Bandibabone, J., Dhokiya, V. *et al.* (2021) Stable high-density and maternally inherited *Wolbachia* infections in *Anopheles moucheti* and *Anopheles demeilloni* mosquitoes. *Current Biology* 31, 2310–2320.e2315.

Wang, J., Li, J., Hou, Y., Dai, W., Xie, R. *et al.* (2021) BastionHub: a universal platform for integrating and analyzing substrates secreted by Gram-negative bacteria. *Nucleic Acids Research* 49, D651–D659.

Wang, S. and Jacobs-Lorena, M. (2013) Genetic approaches to interfere with malaria transmission by vector mosquitoes. *Trends in Biotechnology* 31, 185–193.

Wang, S., Ghosh, A.K., Bongio, N., Stebbings, K.A., Lampe, D.J. and Jacobs-Lorena, M. (2012) Fighting malaria with engineered symbiotic bacteria from vector mosquitoes. *Proceedings of the National Academy of Sciences USA* 109, 12734–12739.

Wang, S., Dos-Santos, A.L.A., Huang, W., Liu, K.C., Oshaghi, M.A. *et al.* (2017) Driving mosquito refractoriness to *Plasmodium falciparum* with engineered symbiotic bacteria. *Science* 357, 1399–1402.

Wang, Y., Gilbreath, T.M. 3rd, Kukutla, P., Yan, G. and Xu, J. (2011) Dynamic gut microbiome across life history of the malaria mosquito *Anopheles gambiae* in Kenya. *PLoS ONE* 6, e24767.

Welch, M., Govindarajan, S., Ness, J.E., Villalobos, A., Gurney, A. *et al.* (2009) Design parameters to control synthetic gene expression in *Escherichia coli*. *PLoS ONE* 4, e7002.

Wilke, A.B. and Marrelli, M.T. (2015) Paratransgenesis: a promising new strategy for mosquito vector control. *Parasites & Vectors* 8, 342.

Wright, O., Delmans, M., Stan, G.B. and Ellis, T. (2015) GeneGuard: a modular plasmid system designed for biosafety. *ACS Synthetic Biology* 4, 307–316.

Yoshida, S., Ioka, D., Matsuoka, H., Endo, H. and Ishii, A. (2001) Bacteria expressing single-chain immunotoxin inhibit malaria parasite development in mosquitoes. *Molecular and Biochemical Parasitology* 113, 89–96.

17 Transgenic Approaches for Sterile Insect Control of Dipteran Livestock Pests and Lepidopteran Crop Pests

Maxwell J. Scott[1]*, Neil I. Morrison[2] and Gregory S. Simmons[3]

[1]*Department of Entomology and Plant Pathology, North Carolina State University, Campus Box 7613, Raleigh NC 27695-7613, USA;* [2]*Oxitec Limited, 71 Milton Park, Oxford, OX14 4RQ United Kingdom;* [3]*Animal Plant Health and Inspection Service, Plant Protection and Quarantine, Science and Technology, United States Department of Agriculture, Salinas, California, United States of America*

17.1 A Brief History of Using the Sterile Insect Technique for Controlling Populations of Agricultural Pests

The Sterile Insect Technique (SIT) is now an established component of a number of integrated approaches to insect pest control (Klassen *et al.*, 2021). The paradigm of this method of pest control has been the eradication of the New World screwworm fly, *Cochliomyia hominivorax* (Coquerel), from the southern USA, Mexico and Central America (Scott *et al.*, 2017; Vargas-Teran *et al.*, 2021). *C. hominivorax* is a devastating pest of warm-blooded animals (Knipling, 1960; Alexander, 2006). *C. hominivorax* females lay their eggs in open wounds or a natural orifice. The hatched larvae then feed on the animal's living tissue. Animals with severe infestations may die if untreated. However, most cases are less severe but are economically important as the animal suffers weight loss and carcasses and hides are damaged.

The history of the *C. hominivorax* SIT program has been thoroughly documented.

In brief, Edward Knipling realized that if large numbers of sterile males could repeatedly be released into wild populations, it would eventually eliminate population reproduction and lead to eradication (Bushland *et al.*, 1955; Knipling, 1960). The program initiated by Knipling and his colleague Raymond Bushland began with release of sterilized insects in Florida in the 1950s. *C. hominivorax* are sterilized by exposure to high doses of radiation. The insects do produce functional gametes but are effectively sterile because their offspring inherit chromosomes carrying dominant-lethal mutations. Thus, matings between the released sterile males and the wild females produce no viable progeny. Subsequently, the SIT approach was used to eradicate *C. hominivorax* from all of the USA. However, farmers in Texas faced an ongoing threat of invasion of *C. hominivorax* from Mexico. To alleviate this threat, SIT was used to eradicate *C. hominivorax* from Mexico in a joint program with the government of Mexico (Krafsur *et al.*, 1987). The program was then extended to

---

*Corresponding author, email: mjscott3@ncsu.edu

eradicate *C. hominivorax* from all of Central America (Wyss, 2000). To prevent re-infestation from South America, sterilized *C. hominivorax* are being constantly released in a 'buffer zone' along the Panama–Colombia border (Scott *et al*., 2017). To maintain releases of *C. hominivorax* in the buffer zone, a mass rearing facility was built in Pacora, Panama, and is jointly run by USDA-APHIS and the Ministry of Agriculture in Panama; these two agencies form the US–Panamanian Commission for the Eradication and Prevention of Screwworms (COPEG).

It is important to note that great success of the *C. hominivorax* SIT program was achieved, although both sterile males and females were released in the field (Wyss, 2000). From the outset, Knipling appreciated that SIT would be more effective if only males were released, as the males would mate with wild females in the release zone rather than the co-released sterile females (Knipling, 1960). Indeed there is experimental evidence that SIT used against the Mediterranean fruit fly is significantly more effective if only males are released (McInnis *et al*., 1994; Rendon *et al*., 2004). However, until recently the *C. hominivorax* eradication program has not had the means to mass-separate males from females. Consequently, very high doses of radiation are used to ensure that the females are 100% sterile (Crystal, 1979), which reduces male longevity and mating competitiveness, decreasing the efficiency of SIT.

Over a period of more than 20 years Max Whitten and Geoff Foster championed the use of fertile males that carry a field female-killing system as an alternative to the classical SIT approach of releasing sterilized males (Whitten and Foster, 1975, Foster *et al*., 1993). A strain of the Australian sheep blowfly *Lucilia cuprina* was developed that contained translocations of eye-colour genes to the Y chromosome. Both males and females were homozygous for loss of function mutations in the eye-colour genes, but males had wild-type eye-colour due to the translocated wild-type genes (Foster *et al*., 1993). In a field trial, males and females were released over Flinders Island. As the females were blind, they did not survive for long in the field. The males passed on the 'field female-killing' genes to their female offspring. The trial was successful in significantly reducing the population of *L. cuprina*. However, due to problems in mass-rearing the strain (breakdown of translocations, semi-sterility) and declining wool price, the trial was never extended to mainland Australia.

Current lepidopteran SIT programs, targeting the pome fruit pest codling moth *Cydia pomonella*, the citrus pest false codling moth *Thaumatotibia leucotreta* and the cotton pest pink bollworm, *Pectinophora gossypiella*, recently eradicated from North America (Staten and Walters, 2021), still rely on bi-sex releases (Marec and Vreysen, 2019). However, there is not a consensus among moth SIT workers that development of single-sex male release strains is necessary for increased effectiveness of the SIT in Lepidoptera, as a large number of female sterile moths released in the field will, by emission of sex pheromones, interfere with mating between wild male and females moths, thereby providing control by mating disruption (Stringer *et al*., 2013). For the cactus moth *Cactoblastis cactorum*, one SIT study found that co-releasing males and females may even enhance the pest-suppression effect, relative to male-only releases (Hight *et al*., 2005).

For the codling moth, an area-wide operational intervention program was initiated in 1992 (Bloem and Bloem, 2000; Simmons *et al*., 2021). A mass-rearing facility was constructed which had a production capacity of 15 million moths per week in 2002 (Marec and Vreysen, 2019; Simmons *et al*., 2021). Excellent results have been obtained in the more than 20 years since operations were started in the first release zone. Insecticide use has been reduced by 96% from 2.5 kg/ha in 1991 to 0.1 kg/ha in 2016, and the proportion of orchards with less than the economic threshold of 0.2% damage, or no detectable level of codling moth damage, at harvest increased from 42% in 1995 to more than 90% in 2015.

Pink bollworm, formerly the major lepidopteran pest of cotton in the southwestern USA and northern Mexico, was successfully controlled by SIT for more than 40 years. The SIT program was originally conceived as

a preventive release strategy to keep the large area of cotton production (> 500,000 ha) in the San Joaquin Valley of California free from pink bollworm (Simmons *et al*., 2021). In 2001, this was expanded into a large, area-wide campaign against the pink bollworm. The aim was regional eradication of the pest using a combination of tactics including mating disruption, regional widespread planting of genetically modified cotton expressing the *Bt* toxin, regulated uniform crop destruction and SIT with release of as many as 200 million sterile moths per week (Henneberry and Naranjo, 1998; Tabashnik *et al*., 2010, 2021; Simmons *et al*., 2021; Staten and Walters, 2021). By 2012, no detections of larvae or moths were recorded in any program areas and by 2018 eradication of pink bollworm was declared for all USA and Mexican program areas (Simmons *et al*., 2021; Staten and Walters, 2021).

17.1.1   Male-only releases

Inspired by the success of the *C. hominivorax* SIT program, this approach has been used to eradicate or suppress populations of other dipteran agricultural pests, particularly tropical tephritid fruit flies (see Handler and Schetelig, Chapter 21, this volume). For example, SIT was used to eradicate the Mexican fruit fly and West Indian fruit fly from several Mexican states, Queensland fruit fly from Western Australia and the melon fly from Okinawa, Japan (Enkerlin, 2021; Klassen *et al*., 2021). The Mediterranean fruit fly *Ceratitis capitata*, or medfly, has been the subject of some of the largest SIT programs, particularly in southern Mexico and northern Guatemala. *C. capitata* males are produced in a mass-rearing facility in Guatemala, which has the capacity to produce over 1 billion sterilized males per week (Rendon *et al*., 2004; Klassen *et al*., 2021). The so-called genetic sexing strain (GSS) is used to obtain only males for release. For *C. capitata*, male-only releases have two advantages. First, as discussed above, this eliminates matings between sterile males and the co-released sterile females. Second, this prevents oviposition-mediated fruit stings by released sterile females that lead to crop spoilage. GSS female embryos die at high temperature whereas males survive, as they carry a resistant gene on the Y chromosome (Franz *et al*., 2021). This treatment is routinely used to eliminate females in the production facility. The GSS took many years to develop and required the isolation of a temperature-sensitive lethal mutation (mapped to chromosome 5), a Y:chromosome 5 translocation that carried the resistant gene and a system for filtering out male recombinants. Unfortunately, this GSS is specific for *Ceratitis capitata* and cannot be transferred to other species.

GSSs have been developed in Lepidoptera by chromosomal translocations. In silk production, males of the silkworm *Bombyx mori* produce better-quality silk more efficiently (see Sezutsu and Tamara, Chapter 20, this volume), so a means of easily producing males only is potentially valuable. Cocoon-colour and egg-colour genes have been translocated to the female W autosome for visual sexing (Tazima *et al*., 1951; Kimura *et al*., 1971). Female-killing GSS – called balanced-lethal (BL) strains – have been made in which males are *trans*-heterozygous for two sex-linked recessive lethal mutations on the Z chromosome, and females carry one of these mutations on their single W chromosome, translocated with a portion of the Z chromosome (Strunnikov, 1975; Marec, 1991). Because female-killing is induced by crossing to wild-type males, the process of male-only production is inefficient and unwieldy. In the Mediterranean flour moth *Ephestia kuehniella*, a pest of stored products, similar BL strains have been developed, but for female-killing two different strains need to be mated (Marec, 1991; Marec *et al*., 1999). As with the *B. mori* BL strains, this system is too cumbersome for mass-rearing, and is not readily transferred to other species.

17.2   Unaddressed Challenges with Classical SIT Programmes

In contrast to dipteran species, Lepidoptera require much higher levels of ionizing

radiation to obtain full sterility (LaChance, 1967; Marec *et al.*, 2021), with consequent negative impacts on male performance in the field. Molecular mechanisms for the high radio-resistance in Lepidoptera might include an inducible cell recovery system and/or a DNA repair process (Koval, 1996). Nevertheless, the main cause of this difference is thought to reside in the different kinetic organization of chromosomes in these two groups of insects. Dipterans possess typical monocentric chromosomes with kinetic activity restricted to the centromere, whereas lepidopteran chromosomes are essentially holokinetic (Murakami and Imai, 1974; Wolf, 1994). They lack distinct primary constrictions (the centromeres) and their kinetic activity is distributed along most of the chromosome length. The holokinetic (or holocentric) chromosome structure ensures that most radiation-induced breaks do not lead to the loss of chromosome fragments as is typical in species with monocentric chromosomes. It also reduces the risk of lethality caused by the formation of unstable aberrations such as dicentric chromosomes.

The impact of chromosome structure on the biological consequences of radiation-induced lesions and the behaviour of chromosomal aberrations have enabled a variant of SIT to be developed for Lepidoptera. The genetic damage induced by sub-sterilizing doses of radiation is inherited and, in some cases, the F_1 individuals (progeny of irradiated moths) are completely sterile. This phenomenon has been called inherited sterility (IS) and has been extensively studied in pest species (Marec *et al.*, 2021). In principle, IS provides efficiency savings as it allows for release of highly competitive male moths, and multiplication of the fully sterile generation in the field, rather than in the factory. IS has been used to eradicate the painted apple moth *Teia anartoides* after its arrival in New Zealand (Suckling *et al.*, 2007). To prevent increases in fruit damage by release of sub-sterilized females, pupae were sexed by hand to permit male-only release. Use of IS on a larger scale is therefore constrained by the likely need for a GSS, to provide an efficient method of producing male-only cohorts

such that releases of irradiated moths do not cause an increase in eggs laid in the field and consequent crop damage. Moreover, a method of releasing male moths carrying a heritable marker, for easy distinction between sterile and non-sterile progeny, would greatly enhance program monitoring capability.

17.3 Biotechnology Enhanced SIT: an Overview

Methods for germline transformation of many insect species have been developed and are routinely used in laboratories worldwide (see O'Brochta, Chapter 1, this volume). Transgenic technologies can augment the SIT by providing a reliable method for marking released insects and a means for releasing only sterilized males.

17.3.1 Transgenic technologies provide a means for reliably marking released insects

Initially, eye-colour marker genes were used to identify transgenic insects from the mutant host strain (Spradling and Rubin, 1982; Loukeris *et al.*, 1995; Coates *et al.*, 1998; Handler *et al.*, 1998). To use these markers required isolation of recipient eye-colour mutant strains for which the genetic basis of the eye-colour mutation was understood. Expression of the wild-type eye-colour gene would complement the mutation. These markers have largely been replaced with fluorescent protein marker genes, which have the distinct advantage that they can be used with wild-type insect strains (Berghammer *et al.*, 1999; Handler and Harrell, 2001). Specifically with regard to the SIT, there are two advantages of using fluorescent protein marker genes. Firstly, a transgenic strain that expresses a stable fluorescent protein would be of value for surveillance and monitoring of released sterilized insects (Parker *et al.*, 2021; Yan *et al.*, 2021). Currently, mass-reared insects can be detected by adding a dye to the diet or by external application

(Parker *et al.*, 2021). For example, medfly pupae are marked by dusting with a dye, which is transferred to the adult as it emerges. However, marking insects using dyes may not be 100% effective. Further, the dyes are expensive and can be a health hazard. To reliably identify a transgenic insect caught in a trap by fluorescence, it is desirable to use a strain that strongly expresses a stable fluorescent protein (e.g., DsRed). Molecular approaches (e.g., PCR of genomic DNA with DNA primers specific for the marker gene) can provide a sensitive and reliable additional method for distinguishing wild-type from released sterilized transgenic insects. This accuracy of screening would be highly valuable in eradication programs in which detection of a single wild-type insect in an eradicated zone would spark additional releases of sterile insects, which could be costly (Simmons *et al.*, 2011; Walters *et al.*, 2012). A second advantage is that sperm can be marked with a fluorescent protein gene by using a testis-specific promoter to drive expression of the gene and marking of the sperm (Catteruccia *et al.*, 2005; Scolari *et al.*, 2008). For assessing the effectiveness of SIT programs, it is desirable to determine the mating status of wild-type female insects caught in traps. Methods have been developed for some insects for determining if the female has mated with a wild-type male or sterilized release male (for example, analyse progeny if female is alive, measure sperm head lengths). However, these methods are laborious (Vreysen, 2021). Simply screening by fluorescence would provide a rapid means of determining if a trapped female had mated with a transgenic or wild-type male or both.

17.3.2   Transgenic marking: pink bollworm case study

While sterile moth release has proved highly effective, as with other sterile release programs (Simmons *et al.*, 2010, 2021; Morrison *et al.*, 2011) a need exists for a more effective, highly competitive sterile insect. High production and release costs are seen as major limiting factors for expansion of SIT against several key lepidopteran pests (Simmons *et al.*, 2010). In addition to the damaging effects of radiation, several factors impact sterile moth quality, including the effects of mass-rearing, handling, shipping and releases (Carpenter *et al.*, 1997; Bloem *et al.*, 1999; Simmons *et al.*, 2010, 2021). To address these issues for pink bollworm, a program to develop and determine whether transgenic technology could enhance the performance of the sterile moth was initiated in 1998 (Peloquin *et al.*, 2000; Simmons *et al.*, 2007). Other aims of the program included providing quality assurance and biosecurity enhancements.

There were two main goals. The first was to develop a strain that expressed a fluorescent protein to provide a reliable and heritable marker for the release strain of sterile moths. As described in the previous section, transformation with a marker gene would add great value to an operational SIT program. For pink bollworm, the marking system used Calco red dye, which sometimes resulted in sterile moths being mistaken for wild moths (Simmons *et al.*, 2011). Furthermore, incorporating a sterile release strain with a heritable marker into the pink bollworm SIT program could allow the application of a lower dose of radiation, making possible the implementation of an IS strategy into the program. With an IS program, lowering the dose of radiation would result in some reproduction of F_1 progeny in a cotton field. Upon detection of larvae in cotton bolls, without the ability to distinguish sterile F_1 progeny from the release of sterile moths from wild larvae, it would not be possible to implement an IS strategy effectively (Simmons *et al.*, 2007, 2011).

The first fluorescent protein-marked pink bollworm line was also the first successful example of germline transformation in Lepidoptera (Peloquin *et al.*, 2000). However, the green fluorescent protein (GFP), regulated by a *B. mori* actin-3 promoter, was only weakly expressed and sometimes difficult to distinguish from a greenish autofluorescence often seen using florescence microscopy (E. Miller and G. Simmons, personal communication). In 2003, several new

transgenic lines were obtained with constructs comprising regulatory sequences from insect viruses driving expression of the red fluorescent protein, DsRed2. These strains showed strong expression of DsRed2 fluorescence that was easily distinguished from red autofluorescence and, unlike the GFP-expressing strains, transgenic larvae, pupae and adult moths could be confidently distinguished from non-transgenic counterparts (Fig. 17.1i,j) (Table 17.1).

One of these strains, called OX1138B, was made homozygous for the transgene insertion and was tested in the laboratory, semi-field and open releases over a period of several years to determine rearing characteristics, reliability and longevity of the marker, and field performance of the moths. The strain showed similar performance to the untransformed strain of pink bollworm under all test conditions (Simmons et al., 2011) and the fluorescent marker showed good stability in field conditions, in which temperatures regularly exceeded 40°C (Walters et al., 2012). Moreover, molecular detection of the transgene insertion by PCR provided a highly reliable and sensitive secondary screening method. During the production seasons of 2014 and 2015 as a biosecurity measure, the pink bollworm (PBW) program released between 47,000 to 90,000 sterile DsRed2 moths per week in the vicinity of the PBW rearing facility to be distinguishable from and mate with potential escapee wild-type moths and to quantify escapee numbers. With the successful eradication, the work with OX1138B was discontinued by the SIT programme.

17.3.3 Molecular genetic systems for development of male-only strains

As discussed above, male-only releases can significantly improve the efficiency of SIT and, for suppression of many pest targets, can be expected to bypass the need for pre-release irradiation, as the dominant female lethal gene is passed on to the next generation. While GSS can be made by classical genetic approaches this requires a considerable effort to obtain the required components (selectable mutation, Y:autosome translocation) and the strains can be unstable under mass-rearing conditions due to male recombination. Further, a GSS strain developed in one species cannot be transferred to another species. In contrast, molecular genetic technologies offered the promise of developing transgenic sexing strains (TSS) that would be stable during mass rearing and could be applied to more than one species. From the outset it was clear that the genetic sexing system had to be lethal only to females and only under certain conditions. The latter would be necessary for rearing the strain in a factory. Over two decades ago, the first genetic systems were developed and tested in *Drosophila melanogaster* (Heinrich and Scott, 2000; Thomas et al., 2000) (see Morrison, Chapter 23, this volume). The two-component system (Fig. 17.2) employed the tetracycline-dependent transactivator (tTA), a transcription factor that could only bind to its target site (tetO) in the absence of tetracycline (Gossen and Bujard, 1992). tTA is a fusion of the DNA binding domain of the tet repressor from *Escherichia coli* and the transcription activation domain from the HSV1 viral protein VP16. In the system developed in the laboratory of one of us (MJS), transcription of the tTA gene was driven by the female-specific promoter from the *yolk protein 1* gene (Heinrich and Scott, 2000). In the absence of tetracycline, tTA induced expression of the pro-apoptotic cell death gene *head involution defective* (*hid*). High levels of *hid* expression induce widespread cell death (Grether et al., 1995), and as a consequence death of the female fly.

Wimmer and colleagues adapted the system shown in Fig. 17.2 to induce early lethality. Essentially, the system is the same, except that the *yp1* enhancer was replaced with a promoter from one of the cellularization genes that is strongly active in the early embryo (Horn and Wimmer, 2003). In fly development one of the first critical steps is forming cells after several rounds of nuclear division. Some of the genes required for this cellularization process such as *serendipity-alpha* (*sry-alpha*), *nullo*, *slow-as-molasses* (*slam*) and *bottleneck* (*bnk*) are transcribed at
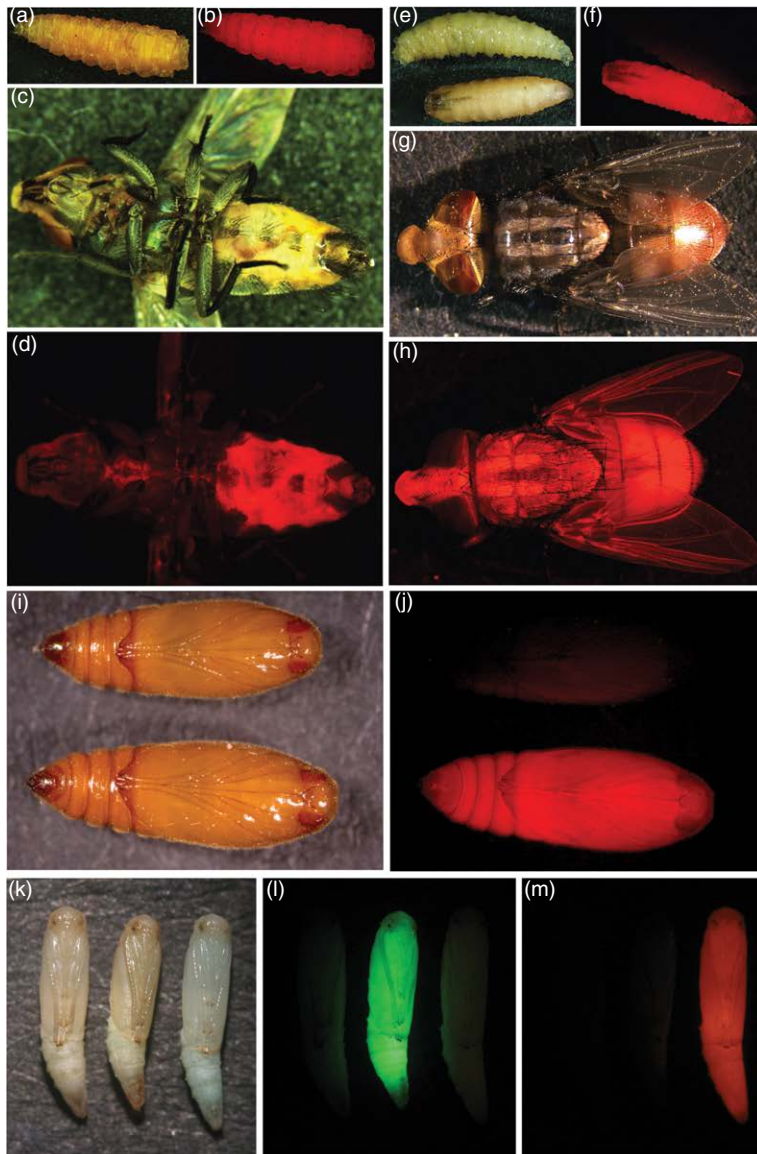
Fig. 17.1. Transgenic insects marked with constitutively expressed fluorescent protein marker genes. Insects photographed under white light or suitable filters. **(a–d)** *Lucilia cuprina* late larvae and young adult carrying a DsRed-Express2 marker gene codon-optimized for calliphorids, driven by the *L. cuprina hsp83* gene promoter. **(e–h)** *Cochliomyia hominivorax* late larvae and young adult, carrying the same marker as described for *L. cuprina*. For comparison, a wild-type larva is shown alongside a transgenic *C. hominivorax* larva. Red fluorescence is clearly visible in young adults within 1 hour of eclosion, but is much more difficult to see in older adults as the cuticle darkens. **(i, j)** Pink bollworm pupa carrying a DsRed2 marker gene, driven by the Hr5*ie1* regulatory sequences from the *Autographica californica* nuclear polyhydrosis virus (AcMNPV), photographed alongside a wild-type pupa. **(k–m)** Diamondback moth pupae carrying the ZsGreen marker gene driven by the *Opie2* promoter fragment from the *Orgyia pseudotsugata* nuclear polyhydrosis virus (middle pupa), the DsRed2 marker gene driven by Hr5*ie1* (right-hand pupa). Photographed alongside a wild-type pupa.

Table 17.1. Summary of transgenic advances for the sterile insect approach in dipteran livestock pests and lepidopteran pests of crops.

Innovation	Potential benefits	Species	Status	References
Heritable marker	1. Reduce false-positives on traps. 2. Permit tracking of sterile progeny in an IS program.	Pink bollworm, *Pectinophora gossypiella*	Irradiated DsRed2-marked moths have undergone large-scale SIT field trials in USA. Fluorescence and molecular detection provide two reliable markers.	Simmons *et al.*, 2011; Walters *et al.*, 2012
		Australian sheep blowfly, *Lucilia cuprina*; and New World screwworm, *Cochliomyia hominivorax*	Strains with strongly expressed ZsGreen and DsRed-Express2 markers have been made. To be evaluated in field conditions.	Concha *et al.*, 2011, 2016; Li *et al.*, 2014
Genetic sterility (plus heritable marker)	1. Replaces need for sterilization by irradiation, which can reduce insect performance. 2. Intrinsic bio-containment as security against accidental releases.	Pink bollworm	Tetracycline-repressible, bi-sex lethal strains developed, including some field cage experiments.	Morrison *et al.*, 2012
Genetic sexing strains (plus heritable marker)	1. SIT by male-only release is potentially much more efficient than bi-sex releases. 2. Per-male production costs reduced. 3. Can replace need for sterilization by irradiation, which can reduce insect performance. 4. Insecticide resistance management. 5. Intrinsic bio-containment as security against accidental releases.	Pink bollworm*;* diamondback moth, *Plutella xylostella*; and fall armyworm, *Spodoptera frugiperda*	Strains developed and characterized, which show tetracycline-repressible, female-specific lethality engineered using sex-alternate splicing sequences	Jin *et al.*, 2013
		L. cuprina and *C. hominivorax*	Strains developed and characterized, which show tetracycline-repressible, female-specific lethality engineered using sex-alternate splicing sequence from *C. hominivorax transformer*.	Li *et al.*, 2014; Yan and Scott, 2015, Concha *et al.*, 2016; Yan *et al.*, 2017; Concha *et al.*, 2020; Yan *et al.*, 2020b

TWO-COMPONENT SYSTEM   ONE-COMPONENT SYSTEM
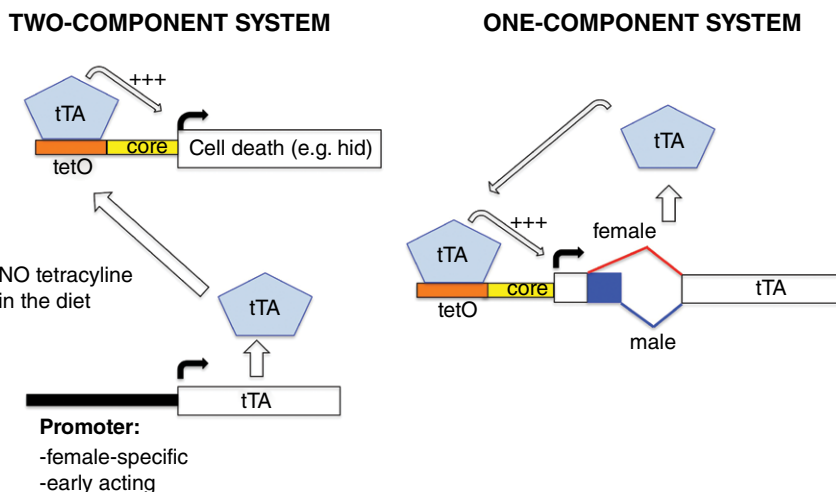
Fig. 17.2. Two- and one-component tetracycline-repressible lethal genetic systems. Central to both one- and two-component systems is the tetracycline-dependent transactivator (tTA), which only binds to DNA and activates transcription if there is no tetracycline in the diet. The two-component systems use female-specific or early-acting promoters to drive tTA expression and activate a cell death gene. The one-component system employs the sex-specifically spliced intron from the *transformer* gene to achieve female-specific expression of tTA. Auto-regulated overexpression of tTA causes lethality, presumably due to interference with general transcription (see Morrison, Chapter 23, this volume). The *transformer* intron can also be included in the hid gene in the two component system to achieve sex-specificity.

high levels at this very early stage of development (Mazumdar and Mazumdar, 2002). In the Horn and Wimmer system, since the promoter is not sex-specific, both males and females die early in development in the absence of tetracycline.

An alternative one-component genetic sterilization system was developed in medfly by Alphey and colleagues (Gong *et al.*, 2005). The system consists of a minimal promoter with upstream tTA binding site driving expression of tTA. In the absence of tetracycline, autoregulation of tTA gene expression will lead to very high levels of production of the tTA transcription factor. This will cause lethality due to 'transcriptional squelching', which is a general interference with gene transcription. Lethality is typically late in development at the larval/pupal stages. However, a transgenic line with just one component is easier to build and would be expected to have fewer fitness costs than a strain with two components. Subsequently, the one-component system was modified to kill only females, yielding male-selecting strains (Fu *et al.*, 2007). Rather than using a female-specific gene enhancer, the sex-specific

intron from the medfly *transformer* (*tra*) gene (Pane *et al.*, 2002) was inserted into tTA open reading frame (Fig. 17.2). *tra* is a key sex-determining gene in a number of insect species (Verhulst *et al.*, 2010) (see Arien *et al.*, Chapter 10, this volume). Only the female *tra* transcript encodes full-length protein. The male *tra* transcript includes additional exons that contain several in-frame stop codons. tTA transcripts containing the *tra* intron were correctly sex-specifically spliced in transgenic medfly (Fu *et al.*, 2007). Thus, in the absence of tetracycline, only females died as only the female transcript encoded tTA protein, which was overexpressed. An identical genetic system has been used to make male-only strains of the olive fruit fly (Ant *et al.*, 2012). Similar tetracycline-repressible female-lethal systems had been developed for *C. hominivorax*, *L. cuprina* and Lepidoptera, and are described later in this review. As larval diet can be a major cost in the mass-rearing facility, the two-component embryo lethal system (Fig. 17.2) was modified to be lethal only to female embryos by incorporation of the medfly *tra* intron into

the *hid* cell death gene (Schetelig and Handler, 2012; Ogaugwu *et al.*, 2013).

While we have discussed the above genetic systems as a means for releasing only sterilized males, an advantage of these systems is that physical sterilization is not required with a dominant repressible lethal system (Heinrich and Scott, 2000; Schliekelman and Gould, 2000; Alphey, 2002). This is because the female offspring of the released fertile males would not develop in the absence of tetracycline. Further, in subsequent generations any females that inherit the transgene would die. Empirical demonstrations of the effectiveness of this approach have been conducted with male-selecting strains for several Diptera (Ant *et al.*, 2012; Leftwich *et al.*, 2014; Li *et al.*, 2021) and Lepidoptera (see below). In brief, these experiments involve repeated releases of an excess of fertile males from the male-selecting strains to established cage populations of the pest species. Over several generations the number of females in the cage is reduced and eventually eliminated.

In addition to providing a stand-alone means of pest suppression, another benefit of releases of males carrying a female-lethal gene would be the introgression of beneficial genes into the target pest population through the survival of male offspring. Modelling has shown this to be a potentially very effective means of managing resistance to insecticides, with introgressed susceptibility genes diluting resistance in the treated population (Alphey *et al.*, 2007, 2009). Such an approach could provide a valuable means of protecting the efficacy of insecticides and *Bt* crops from the threat of pest resistance.

Finally, a major advantage of transgenic insects carrying a tetracycline-repressible lethal system for SIT is that they have a built-in 'fail-safe', as the transgene confers either mortality in all offspring, or half of them (the females): they are termed 'self-limiting' insect strains, as the traits cannot persist in the environment.

17.4 Biotechnology Enhanced SIT: New World Screwworm and the Australian Sheep Blowfly

As discussed above, before elimination by the SIT, *C. hominivorax* was a major livestock pest in the southern USA and Central America. It remains a serious pest in parts of the Caribbean and South America (Vargas-Teran *et al.*, 2021). *L. cuprina* is a major pest of sheep in Australia and New Zealand (Sandeman *et al.*, 2014). *C. hominivorax* and *L. cuprina* are closely related blowflies (McDonagh and Stevens, 2011). Like *C. hominivorax*, *L. cuprina* larvae feed on the animal's living tissue, causing the animal to lose weight and also damaging the hide. In severe cases, flystrike causes death of the sheep (Sandeman *et al.*, 2014).

17.4.1 Germline transformation of *C. hominivorax* and *L. cuprina*

Because of their economic importance, considerable effort was made to develop methods for germline transformation, a prerequisite for developing transgenic strains for genetic control programs. The first success was with *L. cuprina* using a *piggyBac* vector that carried an enhanced green fluorescent protein (EGFP) marker gene (Heinrich *et al.*, 2002). This marker had previously been used by Handler and colleagues for transformation of fruit flies (see Handler and Schetelig, Chapter 21, this volume). Expression of EGFP was driven by the *D. melanogaster polyubiquitin* gene promoter. Subsequently the same system was used to obtain transgenic *C. hominivorax* (Allen *et al.*, 2004). The lines that were obtained showed strong evidence of position effects, with line-specific patterns of expression. This suggests that either the constitutive *polyubiquitin* promoter is being repressed in some tissues or the promoter has a low activity in blowflies and strong expression is dependent upon the presence of nearby transcription enhancers. Consistent with the latter explanation, it was often difficult to identify transgenic *L. cuprina*, as the fluorescence due to the EGFP marker was scarcely above background autofluorescence. Consequently, a much stronger marker was developed that used the *L. cuprina hsp83* promoter to drive expression of ZsGreen or DsRed fluorescent protein genes (Concha *et al.*, 2011; Li *et al.*, 2014).

Transgenic individuals were readily distinguished from wild-type, which was a major advance (Fig. 17.1a–d) (Table 17.1). A helper plasmid that used the *Lchsp83* gene promoter to drive expression of the *piggyBac* transposase also appeared to boost transformation efficiency. Transgenic *C. hominivorax* have been obtained using *piggyBac* vectors that carry either the Lchsp83-ZsGreen or Lchsp83-DsRed-Express2 marker genes. As in *L. cuprina*, the marker genes are strongly expressed at all stages in *C. hominivorax* (Fig. 17.1e–h). Flies that strongly express stable fluorescent proteins should be readily distinguished from wild-type flies caught in field traps. A high priority is to perform experiments to determine the stability of the marker under laboratory and field conditions, similar to what has been done with transgenic pink bollworm (see below).

17.4.2 Development of transgenic sexing strains of *C. hominivorax* and *L. cuprina*

In general, it appears that in order to assemble tetracycline-repressible female lethal systems that function very efficiently it is better to use components (promoter, sex-specific splicing, cell death) from the targeted species or a close relative (Schetelig and Handler, 2012). Thus, to make transgenic strains that have the two-component system that was initially developed in *Drosophila* (Fig. 17.1) (Heinrich and Scott, 2000), it was necessary to isolate a blowfly female-specific promoter and a proapoptotic gene. A *L. cuprina yolk protein* gene promoter was isolated and characterized using a *lacZ* reporter gene assays in transgenic *L. cuprina* (Scott *et al.*, 2011). The promoter was female-specific and regulated by diet. However, the promoter was only active in adult females, which is too late a stage to be useful for an SIT program. Transcripts for the *L. sericata reaper* and *hid* pro-apoptotic genes were identified in a transcriptome assembled from several life history stages (Sze *et al.*, 2012; Edman *et al.*, 2015). The pro-apoptotic activity of these *Lucilia* genes was

initially assessed in transgenic *Drosophila* (Edman *et al.*, 2015). More recently, the *C. hominivorax*, *hid*, *reaper*, *grim* and *sickle* pro-apoptotic genes were identified in the annotated whole genome assembly (Scott *et al.*, 2020).

The sex-specifically spliced first intron from the *transformer* (*tra*) gene is needed to make either the one-component or early-acting two-component female-lethal systems (Fig. 17.1). The *L. cuprina tra* gene (*Lctra*) was isolated and found to be sex-specifically spliced with only the female transcript coding for full-length TRA protein (Concha and Scott, 2009). The presence of multiple copies of 13 nucleotide so-called TRA/TRA2 sites within the sex-specific first intron of the *L. cuprina tra* gene strongly suggested that *tra* splicing is auto-regulated as in the medfly (Pane *et al.*, 2002). TRA/TRA2 sites are known to be important for sex-specific splicing of *dsx* and *fru* transcripts in *Drosophila* (Hoshijima *et al.*, 1991; Heinrichs *et al.*, 1998). The *tra* genes from *C. hominivorax*, *C. macellaria* (secondary screwworm) and *L. sericata* all had a very similar exon–intron organization and relative arrangement of TRA/TRA2 sites as the *L. cuprina tra* gene (Li *et al.*, 2013).

The first *L. cuprina* and *C. hominivorax* TSS contained a single-component auto-regulated tTA gene (Li *et al.*, 2014; Concha *et al.*, 2016) (Table 17.1). Sex-specificity was achieved by using the first intron from the *C. hominivorax tra* gene (Li *et al.*, 2013). The TSS produced equal numbers of males and females on diet with tetracycline but produced only males on diet without tetracycline. Females died at the late larval and pupal stages. Some of the lines showed dominant female lethality: one copy of the transgene was sufficient to kill females. Thus, as discussed above, it would not be necessary to sterilize males from these strains to achieve population suppression. As the *C. hominivorax* TSS were developed within the mass-rearing facility in Panama, they were evaluated using all the standard fitness tests that are routinely used for assessing screwworm strains. Most of the TSS were comparable to the parental Jamaica 06 strain, which is currently used for mass production. We

calculated that the weekly production costs would be reduced by half by using a TSS (Concha *et al.*, 2016). Further savings could be achieved by eliminating the need for radiation treatment and if females died at the embryo or first-instar stage. For these calculations, we conservatively assumed a male-only release would be twice as efficient for population suppression compared with a mixed-sex release.

More recently, two component female embryo TSS have been developed for *L. cuprina* (Yan and Scott, 2015; Yan *et al.*, 2017, 2020a) and *C. hominivorax* (Concha *et al.*, 2020). The TSS produced equal numbers of males and females on diet with tetracycline but produced only males on diet without tetracycline. Females died at the embryo or first-instar stages. The TSS show dominant female lethality. Although we initially assumed two component strains would need to be sterilized by radiation as the components will segregate independently (Yan and Scott, 2015), recent modelling has shown that a fertile release could be efficient for population suppression (Vella *et al.*, 2021).

The initial TSS used the promoter from the *L. sericata bottleneck* (*Lsbnk*) gene to drive tTA expression (Yan and Scott, 2015; Concha *et al.*, 2020). Although the promoter was very active in the early embryo, there was also significant tTA expression in female ovaries in most of the tTA driver lines (Yan *et al.*, 2020a,b). This was problematic, as ideally adult females would be fed a diet free of tetracycline to obtain only male offspring. If not, the antibiotic would be deposited in eggs and could prevent activation of the *hid* gene in developing progeny embryos. One solution was to supply adult females of the TSS a limiting amount of tetracycline (lower dose for only a few days) that was sufficient for egg development but not enough to repress tTA in offspring (Yan and Scott, 2015). Another solution was to search for tTA lines that had little expression in ovaries due to local negative position-effects (Yan *et al.*, 2020b). A third solution was to search for gene promoters that were highly active in the embryo but not in ovaries. We found that promoters from the *L. cuprina nullo* (*Lcnullo*) and *C. macellaria CG14427*

(*CmCG14427*) genes were very active in early embryos but showed no detectable activity in ovaries (Yan *et al.*, 2020a). *L. cuprina* TSS made using tTA drivers with these promoters produced only males on diet without tetracycline and it was not necessary to add tetracycline to the maternal diet. *C. hominivorax* TSS that have the *Lcnullo* and *CmCG14427* tTA driver lines are currently being evaluated. However, the five TSS made earlier using the *Lsbnk*-tTA driver appear quite promising, as all produced only males on diet without tetracycline (Concha *et al.*, 2020). Further, fitness properties that are important for production, such as pupal weight, were comparable to the parental Jamaica 06 strain. Two of the five TSS did not require a maternal supply of tetracycline. One of the TSS was evaluated under mass-rearing conditions using the same 20 l diet trays as in production. On diet without tetracycline, the TSS produced a comparable number of males as the Jamacia 06 strain (Concha *et al.*, 2020). The *C. hominivorax* TSS remain to be evaluated for fitness parameters that are important for field performance (e.g., male competition).

17.5 Biotechnology Enhanced SIT: Lepidoptera

17.5.1 Pink bollworm – a bi-sex self-limiting strain

Beyond the previously discussed marker-only strain, the second goal of the pink bollworm research program was to produce a 'genetically sterile' self-limiting strain that did not need to be irradiated prior to release in the field. Strains of self-limiting pink bollworm were developed that showed tetracycline-repressible bi-sex lethality (Morrison *et al.*, 2012) (Table 17.1), similar to those described in medfly (Gong *et al.*, 2005). Using this one-component genetic sterilization system, tetracycline-repressible lethality in early larvae was engineered in transformed pink bollworm strains. In addition to the traceability of the heritable fluorescent marker, the fact that these insects' progeny

cannot survive without tetracycline in the larval feed, for example on cotton in the field, provides a means of radiation replacement. On cotton plants in the laboratory the larvae of the bi-sex self-limiting strain, OX3402C, die at an early stage, causing minimal damage to bolls (Morrison *et al*., 2012). In less hospitable conditions, for instance in the field, larval death is likely to occur even earlier. Programmed larval death in the absence of the dietary antidote also provides biosecurity and protection against accidental escapes from the mass-rearing facility.

17.5.2   Male-selecting, self-limiting lepidopteran strains

The deficiencies of GSS developed by chromosomal translocations, and the progress achieved in developing TSS in Diptera, indicate that germline transformation is the more promising approach for making such strains in the Lepidoptera. Moreover, the opportunity to develop female-lethal self-limiting strains opens up new opportunities: SIT-like pest suppression without irradiation and management of resistance to other pest management tools.

As described above (and see Handler and Schetelig, Chapter 21, this volume), in the medfly TSS, female-specific expression of a lethal gene is regulated by sex-alternate splicing sequences from the sex determination gene, *transformer* (*tra*). Homologues of *tra* in Lepidoptera are not known. Another gene in the sex determination cascade, *doublesex* (*dsx*), does show differential splicing in males and females (Suzuki *et al*., 2001), and sequence from the pink bollworm *dsx* homologue was therefore used by Jin *et al*. (2013) to regulate female-specific expression of tTAV (Table 17.1). In a *dsx* minigene, the tTAV coding sequence was inserted into a female-transcribed exon, with *tetO* positioned upstream to provide a lethal positive-feedback loop as described in medfly (Fu *et al*., 2007). This exon comprising tTAV coding sequence is spliced out in males but is present in the female transcript. In pink bollworm strains transformed with this construct, females die as larvae when reared off tetracycline, whereas males survive normally. In the presence of tetracycline this lethal phenotype is suppressed (Jin *et al*., 2013). This work has yielded the first lepidopteran strains that provide truly automatic male selection – by simply withholding tetracycline from larval feed – with potential to enhance the pink bollworm SIT program.

The same genetic construct was subsequently used to transform the diamondback moth, *Plutella xylostella*, a globally important pest of Brassicaceae (for example cabbage, broccoli, kale, pak choi, canola) (Fig. 17.1k–m) (Jin *et al*., 2013). Transgenic sexing strains developed showed similar phenotypes to those of the pink bollworm TSS: tetracycline-repressed, tightly controlled female-specific lethality (Table 17.1). Laboratory assessment of two such strains' male mating competitiveness and longevity indicated promise for use in the field (Jin *et al*., 2013), so the lead strain – called OX4319L – was selected for greenhouse studies, in which releases of OX4319L males suppressed caged populations of the diamondback moth, which fell to extinction within a few weeks (Harvey-Samuel *et al*., 2015). This was followed by further greenhouse studies, which demonstrated that releases of OX4319L males diluted resistance to *Bt* (in this case, *Bt*-expressing engineered broccoli), thereby providing enhanced and more sustained pest suppression when both were present, relative to populations treated with only one of OX4319L males or *Bt* broccoli (Harvey-Samuel *et al*., 2015). Following these promising contained studies, OX4319L underwent field trials in upstate New York, USA, in which the modified males showed strong field performance in terms of dispersal and longevity (Shelton *et al*., 2020). Taken together, OX4319L shows promise for delivering benefits to *Brassica* growers in the future and demonstrates an entirely new category of integrated pest management tool: a target-specific pest suppression tool that also protects the efficacy of other tools.

More recently, similar strains have been developed in the fall armyworm, *Spodoptera frugiperda*, which causes particular losses in Brazil, where it readily develops resistance to *Bt* corn varieties (Fatoretto *et al*., 2017), and now

in Africa and other parts of the world where it has invaded in the past decade. A self-limiting strain has undergone early field trials in Brazil and, in 2021, received full biosafety approval from Brazilian regulators (Barroso, 2021).

17.6 Future Directions

Progress to date demonstrates that transgenic technology is readily applied to dipteran livestock pests and Lepidoptera, appears to be easily modified and transferred between species within these groups, and has the potential to deliver major benefits to sustainable food production. Beyond the significant potential benefits of SIT-like programs to suppress damaging pests in a targeted and environmentally friendly manner, this technology has the potential for wider enhancement of integrated pest management strategies through provision of potent resistance management. Transgenic self-limiting insect technology is nearing large-scale application in at least one species of Lepidoptera and is expected to be applied to other species of Diptera and Lepidoptera, but has the potential for wider impact. For other insect orders, such as Coleoptera, development of germline transformation methods and improved knowledge of relevant genetics will inform future development of transgenic approaches to control damaging pests.

Acknowledgements

References

Alexander, J.L. (2006) Screwworms. *Journal of the American Veterinary Medical Association* 228, 357–367.

Allen, M.L., Handler, A.M., Berkebile, D.R. and Skoda, S.R. (2004) *piggyBac* transformation of the New World screwworm, *Cochliomyia hominivorax*, produces multiple distinct mutant strains. *Medical and Veterinary Entomology* 18, 1–9.

Alphey, L. (2002) Re-engineering the sterile insect technique. *Insect Biochemistry and Molecular Biology* 32, 1243–1247.

Alphey, N., Coleman, P.G., Donnelly, C.A. and Alphey, L. (2007) Managing insecticide resistance by mass release of engineered insects. *Journal of Economic Entomology* 100, 1642–1649.

Alphey, N., Bonsall, M.B. and Alphey, L. (2009) Combining pest control and resistance management: synergy of engineered insects with Bt crops. *Journal of Economic Entomology* 102, 717–732.

Ant, T., Koukidou, M., Rempoulakis, P., Gong, H.F., Economopoulos, A. *et al.* (2012) Control of the olive fruit fly using genetics-enhanced sterile insect technique. *BMC Biology* 10, 51.

Barroso, P.A. (2021) Technical opinion for the commercial release of moth *Spodoptera frugiperda* genetically modified strain OX5382G. Document 7350/2021, National Technical Commission on Biosafety (CTNBio), Brasilia, Brazil).

Berghammer, A.J., Klingler, M. and Wimmer, E.A. (1999) A universal marker for transgenic insects. *Nature* 402, 370–371.

Bloem, K.A. and Bloem, S. (2000) SIT for codling moth eradication in British Columbia, Canada. In: Tan, K.H. (ed.) *Area-wide Control of Fruit Flies and Other Insect Pests.* Pernebit Universiti Sains Malaysia, Pulau Pinang, Malaysia, pp. 207–214.

Bloem, S., Bloem, K.A., Carpenter, J.E. and Calkins, C.O. (1999) Inherited sterility in codling moth (Lepidoptera: Tortricidae): effect of substerilizing doses of radiation on insect fecundity, fertility, and control. *Annals of the Entomological Society of America* 92, 222–229.

Bushland, R.C., Lindquist, A.W. and Knipling, E.F. (1955) Eradication of screw-worms through release of sterilized males. *Science* 122, 287–288.

Carpenter, J.E.H., Nelley, N. and Mullinix, B.G. (1997) Effect of substerilizing doses of radiation on sperm precedence in fall armyworm (Lepidoptera: Noctuidae). *Journal of Economic Entomology* 90, 444–448.

Catteruccia, F., Benton, J.P. and Crisanti, A. (2005) An *Anopheles* transgenic sexing strain for vector control. *Nature Biotechnology* 23, 1414–1417.

Coates, C.J., Jasinskiene, N., Miyashiro, L. and James, A.A. (1998) *Mariner* transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. *Proceedings of the National Academy of Sciences USA* 95, 3748–3751.

Concha, C. and Scott, M.J. (2009) Sexual development in *Lucilia cuprina* (Diptera, Calliphoridae) is controlled by the *transformer* gene. *Genetics* 182, 785–798.

Concha, C., Belikoff, E.J., Carey, B.L., Li, F., Schiemann, A.H. and Scott, M.J. (2011) Efficient germline transformation of the economically important pest species *Lucilia cuprina* and *Lucilia sericata* (Diptera, Calliphoridae). *Insect Biochemistry and Molecular Biology* 41, 70–75.

Concha, C., Palavesam, A., Guerrero, F.D., Sagel, A., Li, F. *et al.* (2016) A transgenic male-only strain of the New World screwworm for an improved control program using the sterile insect technique. *BMC Biology* 14, 72.

Concha, C., Yan, Y., Arp, A., Quilarque, E., Sagel, A. *et al.* (2020) An early female lethal system of the New World screwworm, *Cochliomyia hominivorax*, for biotechnology-enhanced SIT. *BMC Genetics* 21, 143.

Crystal, M.M. (1979) Sterilization of screwworm flies (Diptera: Calliphoridae) with gamma rays: restudy after two decades. *Journal of Medical Entomology* 15, 103–108.

Edman, R.M., Linger, R.J., Belikoff, E.J., Li, F., Sze, S.H. *et al.* (2015) Functional characterization of calliphorid cell death genes and cellularization gene promoters for controlling gene expression and cell viability in early embryos. *Insect Molecular Biology* 24, 58–70.

Enkerlin, W.R. (2021) Impact of fruit fly programmes using the sterile insect technique. In*:* Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management*, 2nd edn. CRC Press, Boca Raton, Florida, pp. 979–1006.

Fatoretto, J.C., Michel, A.P., Silva Filho, M.C. and Silva, N. (2017) Adaptive potential of fall armyworm (Lepidoptera: Noctuidae) limits Bt trait durability in Brazil. *Journal of Integrated Pest Management* 8, 17.

Foster, G.G., Weller, G.L., James, W.J., Paschalidis, K.M. and Mckenzie, L.J. (1993) Advances in sheep blowfly genetic control in Australia. In: *Management of Insect Pests: Nuclear and Related Molecular and Genetic Techniques*, 1993 Vienna. International Atomic Energy Agency, pp. 299–312.

Franz, G., Bourtzis, K. and Cáceres, C. (2021) Practical and operational genetic sexing systems based on classical genetic approaches in fruit flies, an example for other species amenable to large-scale rearing for the sterile insect technique. In*:* Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) *Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management*, 2nd edn. CRC Press, Boca Raton, Florida, pp. 575–604.

Fu, G., Condon, K.C., Epton, M.J., Gong, P., Jin, L. *et al.* (2007) Female-specific insect lethality engineered using alternative splicing. *Nature Biotechnology* 25, 353–357.

Gong, P., Epton, M.J., Fu, G., Scaife, S., Hiscox, A. *et al.* (2005) A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nature Biotechnology* 23, 453–456.

Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences USA* 89, 5547–5551.

Grether, M.E., Abrams, J.M., Agapite, J., White, K. and Steller, H. (1995) The *head involution defective* gene of *Drosophila melanogaster* functions in programmed cell death. *Genes & Development* 9, 1694–1708.

Handler, A.M. and Harrell, R.A. 2nd (2001) Transformation of the Caribbean fruit fly, *Anastrepha suspensa*, with a *piggyBac* vector marked with polyubiquitin-regulated GFP. *Insect Biochemistry and Molecular Biology* 31, 199–205.

Handler, A.M., Mccombs, S.D., Fraser, M.J. and Saul, S.H. (1998) The lepidopteran transposon vector, *piggyBac*, mediates germ-line transformation in the Mediterranean fruit fly. *Proceedings of the National Academy of Sciences USA* 95, 7520–7525.

Harvey-Samuel, T., Morrison, N.I., Walker, A.S., Marubbi, T., Yao, J. *et al.* (2015) Pest control and resistance management through release of insects carrying a male-selecting transgene. *BMC Biology* 13, 49.

Heinrich, J.C. and Scott, M.J. (2000) A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. *Proceedings of the National Academy of Sciences USA* 97, 8229–8232.

Heinrich, J.C., Li, X., Henry, R.A., Haack, N., Stringfellow, L. *et al.* (2002) Germ-line transformation of the Australian sheep blowfly *Lucilia cuprina*. *Insect Molecular Biology* 11, 1–10.

Heinrichs, V., Ryner, L.C. and Baker, B.S. (1998) Regulation of sex-specific selection of *fruitless* 5' splice sites by transformer and transformer-2. *Molecular and Cellular Biology* 18, 450–458.

Henneberry, T.J. and Naranjo, S.E. (1998) Integrated management approaches for pink bollworm in the southwestern United States. *Integrated Pest Management Reviews* 3, 31–52.

Hight, S.D., Carpenter, J.E., Bloem, S. and Bloem, K.A. (2005) Developing a sterile insect release program for *Cactoblastis cactorum* (Berg) (Lepidoptera : Pyralidae): Effective overflooding ratios and release-recapture field studies. *Environmental Entomology* 34, 850–856.

Horn, C. and Wimmer, E.A. (2003) A transgene-based, embryo-specific lethality system for insect pest management. *Nature Biotechnology* 21, 64–70.

Hoshijima, K., Inoue, K., Higuchi, I., Sakamoto, H. and Shimura, Y. (1991) Control of *doublesex* alternative splicing by transformer and transformer-2 in *Drosophila*. *Science* 252, 833–836.

Jin, L., Walker, A.S., Fu, G., Harvey-Samuel, T., Dafa'alla, T.H. *et al.* (2013) Engineered female-specific lethality for control of pest Lepidoptera. *ACS Synthetic Biology* 2, 160–166.

Kimura, K., Harada, C. and Aoki, H. (1971) Studies on the W translocation of yellow blood gene in the silkworm (*Bombyx mori*). *Japanese Journal of Breeding* 21, 199–203.

Klassen, W., Curtis, C.F. and Hendrichs, J. (2021) History of the sterile insect technique. In*:* Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management*, 2nd edn. CRC Press, Boca Raton, Florida, pp. 1–44.

Knipling, E.F. (1960) The eradication of the screw-worm fly. *Scientific American* 203, 54–61.

Koval, T.M. (1996) Moths: myths and mysteries of stress resistance. *Bioessays* 18, 149–156.

Krafsur, E.S., Whitten, C.J. and Novy, J.E. (1987) Screwworm eradication in North and Central America. *Parasitology Today* 3, 131–137.

LaChance, L.E. (1967) The induction of dominant lethal mutations in insects by ionizing radiation and chemicals - as related to the sterile-male technique of insect control. In*:* Wright, J.W. and Pal, R. (eds) *Genetics of Insect Vectors of Disease*. Elsevier, Amsterdam, The Netherlands, pp. 617–650.

Leftwich, P.T., Koukidou, M., Rempoulakis, P., Gong, H.-F., Zacharopoulou, A. *et al.* (2014) Genetic elimination of field-cage populations of Mediterranean fruit flies. *Proceedings of the Royal Society B: Biological Sciences* 281, 20141372.

Li, F., Vensko, S.P. 2nd, Belikoff, E.J. and Scott, M.J. (2013) Conservation and sex-specific splicing of the *transformer* gene in the calliphorids *Cochliomyia hominivorax*, *Cochliomyia macellaria* and *Lucilia sericata*. *PLoS ONE* 8, e56303.

Li, F., Wantuch, H.A., Linger, R.J., Belikoff, E.J. and Scott, M.J. (2014) Transgenic sexing system for genetic control of the Australian sheep blow fly *Lucilia cuprina*. *Insect Biochemistry and Molecular Biology* 51, 80–88.

Li, F., Yamamoto, A., Belikoff, E.J., Berger, A., Griffith, E.H. and Scott, M.J. (2021) A conditional female lethal system for genetic suppression of the global fruit crop pest *Drosophila suzukii*. *Pest Management Science* 77, 4915–4922.

Loukeris, T.G., Livadaras, I., Arca, B., Zabalou, S. and Savakis, C. (1995) Gene transfer into the medfly, *Ceratitis capitata*, with a *Drosophila hydei* transposable element. *Science* 270, 2002–2005.

Marec, F. (1991) Genetic control of pest Lepidoptera: construction of a balanced lethal strain in *Ephestia kuehniella*. *Entomologia Experimentalis et Applicata* 61, 271–283.

Marec, F. and Vreysen, M.J.B. (2019) Advances and challenges of using the sterile insect technique for the management of pest Lepidoptera. *Insects* 10, 371.

Marec, F., Kollárová, I. and Pavelka, J. (1999) Radiation-induced inherited sterility combined with a genetic sexing system in *Ephestia kuehniella* (Lepidoptera: Pyralidae). *Annals of the Entomological Society of America* 92, 250–259.

Marec, F., Bloem, S. and Carpenter, J.E. (2021) Inherited sterility in insects. In*:* Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management*, 2nd edn. CRC Press, Baco Raton, Florida, pp. 163–200.

Mazumdar, A. and Mazumdar, M. (2002) How one becomes many: blastoderm cellularization in *Drosophila melanogaster*. *Bioessays* 24, 1012–1022.

McDonagh, L.M. and Stevens, J.R. (2011) The molecular systematics of blowflies and screwworm flies (Diptera: Calliphoridae) using 28S rRNA, COX1 and EF-1alpha: insights into the evolution of dipteran parasitism. *Parasitology* 138, 1760–1777.

McInnis, D.O., Tam, S., Grace, C. and Miyashita, D. (1994) Population suppression and sterility induced by variable sex ratio, sterile insect releases of *Ceratitis capitata* (Diptera: Tephritidae) in Hawaii. *Annals of the Entomological Society of America* 87, 231–240.

Morrison, N.I., Franz, G., Koukidou, M., Miller, T.A., Saccone, G. *et al.* (2011) Genetic improvements to the sterile insect technique for agricultural pests. *Asia-Pacific Journal of Molecular Biology and Biotechnology* 18, 275–295.

Morrison, N.I., Simmons, G.S., Fu, G., O'Connell, S., Walker, A.S. *et al.* (2012) Engineered repressible lethality for controlling the pink bollworm, a lepidopteran pest of cotton. *PLoS ONE* 7, e50922.

Murakami, A. and Imai, H.T. (1974) Cytological evidence for holocentric chromosomes of the silkworms, *Bombyx mori* and *B. mandarina* (Bombycidae, Lepidoptera). *Chromosoma* 47, 167–178.

Ogaugwu, C.E., Schetelig, M.F. and Wimmer, E.A. (2013) Transgenic sexing system for *Ceratitis capitata* (Diptera: Tephritidae) based on female-specific embryonic lethality. *Insect Biochemistry and Molecular Biology* 43, 1–8.

Pane, A., Salvemini, M., Delli Bovi, P., Polito, C. and Saccone, G. (2002) The *transformer* gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. *Development* 129, 3715–3725.

Parker, A.G., Mamai, W. and Maiga, H. (2021) Mass-rearing for the sterile insect technique. In*:* Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management*, 2nd edn. CRC Press, Baco Raton, Florida, pp. 283–316.

Peloquin, J.J., Thibault, S.T., Staten, R. and Miller, T.A. (2000) Germ-line transformation of pink bollworm (Lepidoptera: Gelechiidae) mediated by the *piggyBac* transposable element. *Insect Molecular Biology* 9**,** 323–333.

Rendon, P., McInnis, D., Lance, D. and Stewart, J. (2004) Medfly (Diptera: Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. *Journal of Economic Entomology* 97, 1547–1553.

Sandeman, R.M., Levot, G.W., Heath, A.C., James, P.J., Greeff, J.C. *et al.* (2014) Control of the sheep blowfly in Australia and New Zealand – are we there yet? *International Journal for Parasitology* 44, 879-891.

Schetelig, M.F. and Handler, A.M. (2012) A transgenic embryonic sexing system for *Anastrepha suspensa* (Diptera: Tephritidae). *Insect Biochemistry and Molecular Biology* 42, 790–795.

Schliekelman, P. and Gould, F. (2000) Pest control by the release of insects carrying a female-killing allele on multiple loci. *Journal of Economic Entomology* 93, 1566–1579.

Scolari, F., Schetelig, M.F., Bertin, S., Malacrida, A.R., Gasperi, G. and Wimmer, E.A. (2008) Fluorescent sperm marking to improve the fight against the pest insect *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae). *Nature Biotechnology* 25, 76–84.

Scott, M.J., Atapattu, A., Schiemann, A.H., Concha, C., Henry, R. *et al.* (2011) Organisation and expression of a cluster of *yolk protein* genes in the Australian sheep blowfly, *Lucilia cuprina*. *Genetica* 139, 63–70.

Scott, M.J., Concha, C., Welch, J.B., Phillips, P.L. and Skoda, S.R. (2017) Review of research advances in the screwworm eradication program over the past 25 years. *Entomologia Experimentalis et Applicata* 164, 226–236.

Scott, M.J., Benoit, J.B., Davis, R.J., Bailey, S.T., Varga, V. *et al.* (2020) Genomic analyses of a livestock pest, the New World screwworm, find potential targets for genetic control programs. *Communications Biology* 3, 424.

Shelton, A.M., Long, S.J., Walker, A.S., Bolton, M., Collins, H.L. *et al.* (2020) First field release of a genetically engineered, self-limiting agricultural pest insect: evaluating its potential for future crop protection. *Frontiers in Bioengineering and Biotechnology* 7, 482.

Simmons, G.S., Alphey, L.S., Vasquez, T., Morrison, N.I., Epton, M.J. *et al.* (2007) Potential use of a conditional lethal transgenic pink bollworm *Pectinophora gossypiella* in area-wide eradication or suppression programmes. In*:* Vreysen, M.J.B., Robinson, A.S. and Hendrichs, J. (eds) *Area-wide Control of Insect Pests*. Springer Netherlands, Dordrecht, pp. 119–123.

Simmons, G.S., Suckling, D.M., Carpenter, J.E., Addison, M.F., Dyck, V.A. and Vreysen, M.J.B. (2010) Improved quality management to enhance the efficacy of the sterile insect technique for lepidopteran pests. *Journal of Applied Entomology* 134, 261–273.

Simmons, G.S., McKemey, A.R., Morrison, N.I., O'Connell, S., Tabashnik, B.E. *et al.* (2011) Field performance of a genetically engineered strain of pink bollworm. *PLoS ONE* 6, e24110.

Simmons, G.S., Bloem, K.A., Bloem, S., Carpenter, J.E. and Suckling, D.M. (2021) Impact of moth suppression/eradication programmes using the sterile insect technique or inherited sterility. In*:* Dyck, V.A.,

Hendrichs, J. and Robinson, A.S. (eds) *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management*, 2nd edn. CRC Press, Boca Raton, Florida, pp. 1007–1050.

Spradling, A.C. and Rubin, G.M. (1982) Transposition of cloned *P* elements into *Drosophila* germ line chromosomes. *Science* 218, 341–347.

Staten, R.T. and Walters, M.L. (2021) Technology used by field managers for pink bollworm eradication with its successful outcome in the United States and Mexico. In*:* Hendrichs, J., Periera, R. and Vreysen, M.J.B. (eds) *Area-wide Integrated Pest Management*. *Development and Field Application*. CRC Press, Boca Raton, Florida, pp. 51–92.

Stringer, L.D., Sullivan, N.J., Sullivan, T.E.S., Mitchell, V.J., Manning, L.-a.M. *et al.* (2013) Attractiveness and competitiveness of irradiated light brown apple moths. *Entomologia Experimentalis et Applicata* 148, 203–212.

Strunnikov, V. (1975) Sex control in silkworms. *Nature* 255, 111–113.

Suckling, D., Barrington, A., Chhagan, A., Stephens, A., Burnip, G. *et al.* (2007) Eradication of the Australian painted apple moth *Teia anartoides* in New Zealand: trapping, inherited sterility, and male competitiveness. In*:* Vreysen, M., Robinson, A. and Hendrichs, J. (eds) *Area-Wide Control of Insect Pests*. Springer, pp. 603–615.

Suzuki, M.G., Ohbayashi, F., Mita, K. and Shimada, T. (2001) The mechanism of sex-specific splicing at the *doublesex* gene is different between *Drosophila melanogaster* and *Bombyx mori*. *Insect Biochemistry and Molecular Biology* 31, 1201–1211.

Sze, S.H., Dunham, J.P., Carey, B., Chang, P.L., Li, F. *et al.* (2012) A *de novo* transcriptome assembly of *Lucilia sericata* (Diptera: Calliphoridae) with predicted alternative splices, single nucleotide polymorphisms and transcript expression estimates. *Insect Molecular Biology* 21, 205–221.

Tabashnik, B.E., Sisterson, M.S., Ellsworth, P.C., Dennehy, T.J., Antilla, L. *et al.* (2010) Suppressing resistance to *Bt* cotton with sterile insect releases. *Nature Biotechnology* 28, 1304–1307.

Tabashnik, B.E., Liesner, L.R., Ellsworth, P.C., Unnithan, G.C., Fabrick, J.A. *et al.* (2021) Transgenic cotton and sterile insect releases synergize eradication of pink bollworm a century after it invaded the United States. *Proceedings of the National Academy of Sciences USA* 118, e2019115118.

Tazima, Y., Ohta, N. and Harada, C. (1951) On the sex determining method by colouring genes of silkworm eggs. I. Induction of translocation between the W and the tenth chromosomes. *Japanese Journal of Breeding* 1, 47–50.

Thomas, D.D., Donnelly, C.A., Wood, R.J. and Alphey, L.S. (2000) Insect population control using a dominant, repressible, lethal genetic system. *Science* 287, 2474–2476.

Vargas-Teran, M., Spradbery, J.P., Hofmann, H.C. and Tweddle, N.E. (2021) Impact of screwworm eradication programmes using the sterile insect technique. In*:* Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management*, 2nd edn. CRC Press, Baco Raton, Florida, pp. 949–978.

Vella, M.R., Gould, F. and Lloyd, A.L. (2021) Mathematical modeling of genetic pest management through female-specific lethality: Is one locus better than two? *Evolutionary Applications* 14, 1612–1622.

Verhulst, E.C., Van De Zande, L. and Beukeboom, L.W. (2010) Insect sex determination: it all evolves around *transformer*. *Current Opinion in Genetics & Development* 20, 376–383.

Vreysen, M.J.B. (2021) Monitoring sterile and wild insects in area-wide integrated pest management programmes. In*:* Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management*, 2nd edn. CRC Press, Boca Raton, Florida, pp. 485–528.

Walters, M., Morrison, N.I., Claus, J., Tang, G., Phillips, C.E. *et al.* (2012) Field longevity of a fluorescent protein marker in an engineered strain of the pink bollworm, *Pectinophora gossypiella* (Saunders). *PLoS ONE* 7, e38547.

Whitten, M.J. and Foster, G.G. (1975) Genetical methods of pest control. *Annual Review of Entomology* 20, 461–476.

Wolf, K.W. (1994) The unique structure of lepidopteran spindles. *International Review of Cytology* 152, 1–48.

Wyss, J.H. (2000) Screwworm eradication in the Americas. *Annals of the New York Academy of Sciences* 916, 186–193.

Yan, Y. and Scott, M.J. (2015) A transgenic embryonic sexing system for the Australian sheep blow fly *Lucilia cuprina*. *Scientific Reports* 5, 16090.

Yan, Y., Linger, R.J. and Scott, M.J. (2017) Building early-larval sexing systems for genetic control of the Australian sheep blow fly *Lucilia cuprina* using two constitutive promoters. *Scientific Reports* 7, 2538.

Yan, Y., Williamson, M.E., Davis, R.J., Andere, A.A., Picard, C.J. and Scott, M.J. (2020a) Improved transgenic sexing strains for genetic control of the Australian sheep blow fly *Lucilia cuprina* using embryo-specific gene promoters. *Molecular Genetics and Genomics* 295, 287–298.

Yan, Y., Williamson, M.E. and Scott, M.J. (2020b) Using moderate transgene expression to improve the genetic sexing system of the Australian sheep blow fly *Lucilia cuprina*. *Insects* 11, 797.

Yan, Y., Schwirz, J. and Schetelig, M.F. (2021) Characterization of the *Drosophila suzukii beta2-tubulin* gene and the utilization of its promoter to monitor sex separation and insemination. *Gene* 771, 145366.

18 Honey Bee Genome Editing

Alison McAfee[1,2,3]*, Judy Li[2,3] and Marianne Otte[4]

[1]*Department of Applied Ecology, North Carolina State University, Raleigh, USA;*
[2]*Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, Canada;* [3]*Michael Smith Laboratories, University of British Columbia, Vancouver, Canada;* [4]*Institute of Evolutionary Genetics, Heinrich Heine University Düsseldorf, Düsseldorf, Germany*

18.1 Introduction

Honey bees (*Apis mellifera*) have been managed by humans for millennia and intrigue around their teeming, intricate societies has led them to become a classic subject for scientific studies. Indeed, their utility as a model organism for subjects such as social behaviour, learning and memory, development and sex determination motivated the sequencing of the honey bee genome in 2006, making it one of the first insect genomes to be sequenced. But understanding how those genes function and interact to give rise to such astoundingly complex traits requires genetic manipulation. Recent advances in honey bee genome editing create exciting opportunities to study mechanisms of social behaviour and the biological processes that govern how eusocial societies function. Honey bees are also essential pollinators for many agricultural operations; therefore, although not yet commercially practised, genetic engineering of honey bees and their symbionts could have future industrial applications and debate around such applications is pertinent. Here, we provide an overview of honey bee genome editing, including germline and somatic mutagenesis methods, recent research topics and findings, potential industrial applications, challenges and ethical considerations.

18.2 The Value of Honey Bees

Honey bees (*Apis mellifera*) are famous for their complex social architecture and dance language. Indeed, the decoding of the waggle dance was a landmark discovery, earning Karl von Frisch the 1973 Nobel Prize in Physiology. Today, honey bees are a valuable model for social behaviour, including topics such as disease transmission, collective immune defences, division of labour, as well as physiological processes such as ageing and learning. Their tendency to collect plant products (nectar, pollen and resin) and water from distant sources and bring the materials back to the hive also makes them an ideal biomonitor for environmental pollutants (Smith *et al.*, 2019; McAfee *et al.*, 2020). Despite great interest in their behaviour, physiology and ecosystem services, surprisingly little is known about the specific genetic mechanisms underlying these traits.

---

*Corresponding author, email: alison.n.mcafee@gmail.com

Though they are scientifically valuable as a model system, honey bees are also economically important livestock, mainly through their honey production and pollination services. Humans have managed beehives for thousands of years (Winston, 1991), first for their honey and wax, and more recently for agricultural pollination (Klein *et al.*, 2006). Estimates place the worldwide value of crop pollination by animals at US\$235–577 billion annually (inflated to the 2015 value) (Potts *et al.*, 2016) and bee species are the biggest contributors to this figure (Klein *et al.*, 2006). Despite being non-native to North America, South America and Australia, even in non-agricultural landscapes honey bees are the most frequent floral visitor worldwide, owing to their proliferous colonies, generalist foraging strategy and global distribution (Hung *et al.*, 2018). In the USA alone, the economic contribution of the beekeeping industry is estimated at up to US\$34 billion annually, mainly through improved crop yields (Jordan *et al.*, 2021). Native pollinator declines increase reliance on managed pollinators for agricultural pollination and, worryingly, annual honey bee colony losses have been unsustainably high (Currie *et al.*, 2010; Potts *et al.*, 2010; Goulson *et al.*, 2015; Kulhanek *et al.*, 2017). In some regions, apiculture as a practice has also been declining (Neumann and Carreck, 2010). Tools that improve the health and profitability of beehives are therefore needed and the potential for genetic engineering to begin to fill that gap is a topic that cannot be ignored.

18.3 Overview of Honey Bee Genome Editing

So far, advances in honey bee genome editing have been driven by basic research questions, rather than industrial applications, such as method development (Kohno *et al.*, 2016; Otte *et al.*, 2018; Hu *et al.*, 2019), studying mechanisms of sex determination (Roth *et al.*, 2019; Wang *et al.*, 2021), taste perception (Değirmenci *et al.*, 2020), male development and cuticle pigmentation

(Kohno and Kubo, 2018; Nie *et al.*, 2021) and neural development of the olfactory system (Chen *et al.*, 2021). Many of these questions could theoretically be investigated using RNA interference (RNAi); however, its transient nature, difficulties in targeting specific tissues and widespread off-target effects documented in honey bees are problematic (Flenniken and Andino, 2013; Nunes *et al.*, 2013). In honey bees, double-stranded RNA is a particularly important immune elicitor, as it is even shared between individuals in the hive to create immunity ripple effects (Maori *et al.*, 2019). Therefore, the technique cannot easily be used to investigate other immune processes, due to confounding effects of the treatment, and even non-immune processes are often affected (Nunes *et al.*, 2013). Genome editing offers a crisp approach for mechanistic studies, including studying the effects of specific mutations, which is not possible with RNAi.

While transposable elements once dominated the field of insect genome editing (Atkinson *et al.*, 2001), more recently the CRISPR/Cas9 system has been favoured (Xu *et al.*, 2019). As a highly efficient and flexible tool, the advent of CRISPR/Cas9 has had a massive impact on the field of genome editing in general (see Concha and Papa, Chapter 7, this volume). Previously, generating knockout honey bees was not routinely feasible. There is no high-throughput method of introducing molecular gene editing machinery into bee eggs, and the success rate of generating knockouts by classic methods was prohibitively low to be paired with manual microinjection.

Semi-random gene insertion using transposable elements, like the *piggyBac* system, was therefore one of the first methods explored for manipulating the honey bee genome (Schulte *et al.*, 2014). However, the questions that can be addressed by this method are limited, and since the *piggyBac* transposon insertion site can be any TTAA motif, the transposable element itself can cause unintended gene disruptions. As the CRISPR/Cas9 gene editing technology becomes increasingly refined, enabling knockouts, knock-ins and gene drives – genetic modifications that can be self-copied from a modified chromosome into a wild-type chromosome

(Brossard *et al.*, 2019; Moon *et al.*, 2019) – the scope of mechanistic research questions that can be answered will only continue to expand.

18.4 Germline Gene Editing

The social structure in honey bee colonies presents both practical challenges and unique opportunities. Colonies are typically made up of a single reproductive queen, tens of thousands of sterile female workers, and between zero and several thousand drones (reproductive males), depending on the time of year (Winston, 1991). This means that, to achieve heritable genetic modification, either queen or drone gametes must acquire a stable genetic change and not the more abundant workers, which have gametes but do not normally lay eggs. Moreover, female honey bees are diploid, whereas drones are haploid, inheriting one of the queens' alleles. Drones, therefore, can produce grandsons but not sons, and in the absence of a gene drive system, genome-edited drones can contribute only one modified allele to their daughters. A genome-edited queen, likewise, can produce hemizygous drones or heterozygous females, assuming she is inseminated with wild-type semen (Fig. 18.1).

18.4.1 Engineering queens

Queens store all the sperm they need for their lifetime in a sperm storage organ, the spermatheca, and can live for several years, whereas drones live for several weeks and die soon after ejaculation. A single fertile queen can theoretically produce hundreds of thousands of progeny carrying a genetic change over the course of her lifetime. A drone carrying a genetic modification could similarly yield many thousands of progeny if its semen is harvested and used for instrumental insemination of a queen. A queen's long lifespan, high fecundity and potential to produce both sons and daughters with altered genetics has made editing the genome of queens the typically favoured approach.

However, germline gene editing in honey bees can be challenging. Foundational transgenic techniques generally involve microinjecting molecular machinery into embryos or eggs very early in development (see O'Brochta, Chapter 1, this volume), but a female egg's destiny as a sterile worker or reproductive queen is governed by the diet the resulting larva is fed (Kucharski *et al.*, 2008). Workers diligently care for larvae by feeding them a series of glandular secretions until the larvae cease feeding and begin to pupate. Despite specialized management techniques that induce workers to rear certain larvae into queens (Büchler *et al.*, 2013), workers are prone to reject manipulated larvae, making queen rearing from microinjected eggs a tricky endeavour. Furthermore, genome editing efficiency varies depending on the gene being targeted or inserted, as well as the number of nuclei present in the zygote; therefore, in practice, the genome-edited individual is usually a genetic mosaic of cells carrying wild-type and edited alleles. Nevertheless, editing the genome of queens has been the most common approach for germline genome editing thus far, although manipulating semen, rather than oocytes, is also a possibility that has been preliminarily investigated (Robinson *et al.*, 2000).

18.4.2 Methods of introducing genome editing machinery to oocytes

Molecular components for genome editing are most often introduced into oocytes by microinjection (Fig. 18.2). Honey bee eggs can be harvested by caging queens on specialized egg-collection cassettes with removable plastic plugs or film, on which the eggs are laid, or by caging queens on empty comb, which necessitates the delicate manual removal of eggs from their cells. Once collected, the eggs should be kept warm (about 33°C, the core temperature of a beehive) to minimize developmental abnormalities. Otte *et al.* (2018) found that injection into the first anterior third of the egg yielded the highest germline integration efficiency (Fig. 18.2a), presumably because that region of the
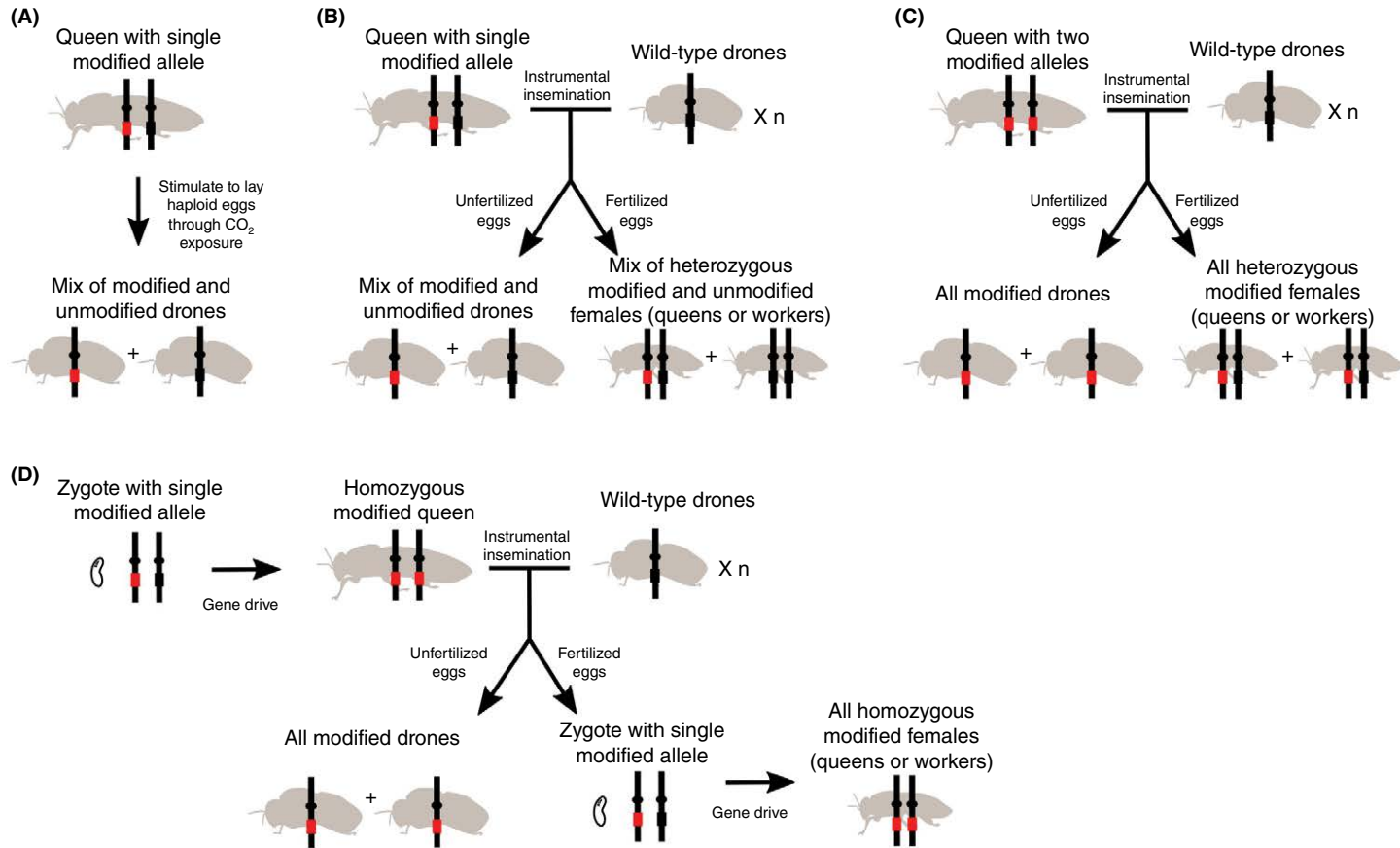
Fig. 18.1. Schematic of inheritance patterns from genome-edited queens. Modified alleles are red and wild-type alleles are black. (A) Inheritance pattern from a heterozygous virgin queen. Virgin queens can be stimulated to lay unfertilized (drone) eggs by carbon dioxide exposure. A queen fully heterozygous for a modification will produce 50% modified and 50% unmodified drones, but in practice, full heterozygosity is seldom obtained (more likely, the bee is a genetic mosaic). **(B)** Inheritance pattern from a heterozygous queen inseminated with wild-type semen. **(C)** Inheritance pattern from a queen with two modified alleles. Genome editing tools do not necessarily create the same genetic change every time, so even if both alleles are modified, the queen may not be homozygous. **(D)** Gene drives enable propagation of a genetic change, producing homozygous individuals from initially heterozygous zygotes. (Bee silhouettes adapted from McAfee *et al*. (2019), CC-BY 4.0 license.)
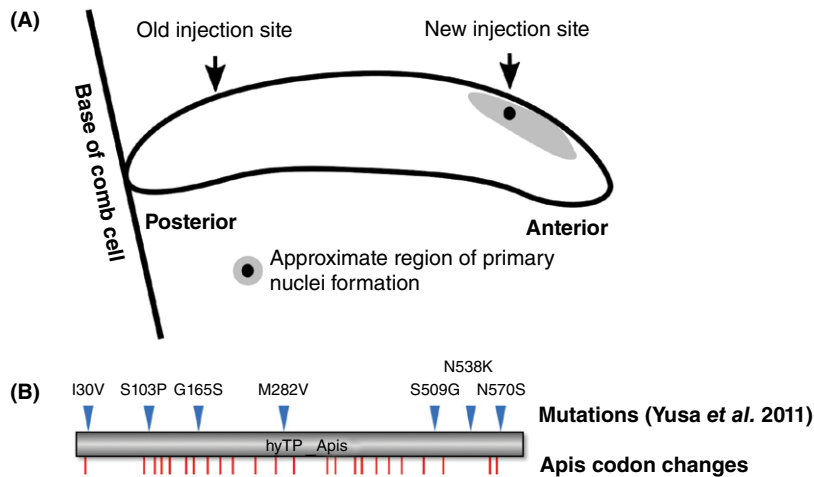
Fig. 18.2. Improvements to transformation efficiency. (A) Injecting into the first anterior third of the egg improved transformation rates (Otte *et al*., 2018). **(B)** Using an altered transposase sequence including mutations reported in Yusa *et al*. (2001) and altered codon usage to match *Apis mellifera* sequences also improved transformation rates (Otte *et al*., 2018). (Figure contributed by Martin Beye.)

egg also contains markers of the germ cells and is the general region of zygote formation in very young eggs (Dearden, 2006). These findings were corroborated by Hu *et al*. (2019), who compared anterior and posterior injections of CRISPR/Cas9 machinery. Eggs typically hatch after 72 h and are normally collected for injection between 1 h and 4 h after being laid, to ensure that the genome editing machinery is introduced at the syncytial blastoderm stage, before membranes form to segregate the nuclei (Fleig and Sander, 1986). Egg hatching rates between 5% and 45% have been reported using this technique, with substantial mortality occurring due to trauma (Schulte *et al*., 2014; McAfee, 2018).

Microinjecting eggs with DNA constructs and other components to facilitate genomic integration or gene editing is the standard technique for germline genome editing, but Robinson *et al*. (2000) identified an alternative strategy for DNA introduction. The researchers demonstrated that linearized DNA containing an expression cassette can be mixed with drone semen and then used to instrumentally inseminate an unmated queen. This results in passage of the foreign DNA into the egg, as evidenced by PCR screening, and gene expression was confirmed by fluorescence imaging. Although

no evidence of genomic integration was identified in their study, the DNA construct was stably carried and expressed in larvae for up to three queen generations (Table 18.1), and in larvae from a single queen for up to 6 months. The authors speculated that the DNA likely gains entry to the oocyte by binding to sperm and being subsequently carried into the egg as the sperm enters through the micropyle, which is a small channel in the anterior end of the egg (unlike mammalian sperm, insect sperm do not require an acrosomal reaction to enter eggs) (Snodgrass, 1910). It is not clear if proteins, such as Cas9, could enter the egg by the same mechanism, but this technique could theoretically be used to introduce DNA constructs – including genes which, when expressed, could facilitate genomic integration – without the need for microinjection. Despite these advantages, this approach appears to have largely not been pursued.

A novel method for introducing gene editing components into germ cells is to capitalize on naturally occurring ligand-mediated transduction of materials from insect haemolymph into the oocytes. Chaverra-Rodriguez *et al*. (2018) developed a technology, ReMOT (receptor-mediated ovary transduction of cargo) (see Terradas *et al*., Chapter 6,

Table 18.1. Inheritance of linearized DNA introduced to queens via sperm mixing. (Original data reported in Robinson *et al*., 2000.)

First generation		
Queens inseminated with sperm/plasmid mixture	Queens yielding progeny	Queens with progeny carrying cassette
42	25	8
Second generation		
Queens reared from cassette-carrying larvae	Queens yielding progeny	Queens with progeny carrying cassette
18	13	3
Third generation		
Queens reared from cassette-carrying larvae	Queens yielding progeny	Queens with progeny carrying cassette
7	6	4

this volume), which enables transfer of Cas9 machinery into mosquito eggs by covalently linking the Cas9 protein to a species-specific peptide sequence that interacts with an oocyte receptor to mediate transport. Injecting adult mosquitoes with this peptide-linked Cas9 led to nearly one in three injected mosquitos becoming germline mutants, and the approach is effective in several mosquito species (Chaverra-Rodriguez *et al*., 2018). While the ReMOT approach has not yet been demonstrated in honey bees, efforts are underway (C.M. Grozinger, Pennsylvania, 2021, personal communication). If successful, this strategy could enable germline mutation while avoiding the high mortality associated with embryonic injection and the tedious process of subsequently rearing those larvae into queens.

18.5 Somatic Gene Editing and Transgene Expression

Germline gene editing is desirable for studying developmental processes or behavioural systems involving multiple related individuals, but somatic genome editing or transgenesis is sufficient, even preferred, for some applications. For example, it may be advantageous to introduce a genetic change in a localized population of cells if that change is expected to have undesirable or extraneous physiological effects stemming from other tissues. Somatic gene editing and transgenesis in honey bees have only been performed on five occasions, either by introducing

genetic material to cells using electroporation (Kunieda and Kubo, 2004; Schulte *et al*., 2013), or by using a baculovirus vector (Ando *et al*., 2007), or by local injection of CRISPR/Cas9 machinery (Sinakevitch *et al*., 2020). One other example of somatic gene editing in honey bees has been reported (Chen *et al*., 2021), but since these mutant bees were produced via embryonic injection, they are likely still germline mosaics and the work is therefore discussed elsewhere.

18.5.1 Electroporation

Kunieda and Kubo (2004) demonstrated that plasmids can be introduced to worker bee brain cells *in vivo* using electroporation, and reporter gene expression was subsequently detected. The researchers introduced the expression cassette by injecting the cassette-containing solution in the brain between two electrodes inserted into the optic lobes or mushroom bodies. They subsequently administered electrical pulses at different voltages (10–90 V) and found that higher voltages yielded higher gene transfer rates. Interestingly, cassette expression was strongest near the anode position (the positive electrode), presumably because DNA is negatively charged and will migrate to this pole under an electric field. Although the procedure was traumatic, the surviving bees were still able to walk freely for up to 1 day, at which time their tissue was analysed.

Schulte *et al.* (2013) used similar brain electroporation techniques to characterize honey bee promotor sequences. While neither of these electroporation studies actually aimed to achieve genomic integration of transgenes, they did demonstrate the feasibility of introducing nucleic acids into adult worker brain cells *in vivo*. Cells could, in theory, be co-transfected with a cassette-containing plasmid and transposase to enable genome integration. The foundational work of Schulte *et al.* and Kunieda and Kubo also identified several promoter sequences that can be used for ubiquitous (*Am-actin5c*, an actin promoter; *6xP3*, a universal insect promoter; and *CMV*, a cytomegalovirus promoter), targeted (*elp2l*, a neuron-specific promoter) and inducible (*Am-hsp83*, inducible by cold-shock) transgene expression (Table 18.2). Schulte *et al.* (2013) also confirmed functionality of the *Am-hsp70* promoter and Robinson *et al.* (2000) confirmed functioning of the *Drosophila hsp70* promoter in honey bees, but neither could verify their inducibility.

18.5.2   Baculovirus systems

Viruses are masters at penetrating the natural cellular barriers to achieve nucleus entry. Baculoviruses are common vectors used for transient gene expression, as they do not normally integrate their DNA into the host genome (although this can occur sporadically) (Mansouri *et al.*, 2016). Ikeda *et al.* (2011) injected queen pupae with a baculovirus containing a reporter gene and observed that adult queens retained transgene expression in their fat bodies, measured at 4–5 days post-injection. While reporter expression was not detected in the ovaries, alterations to the injection location and using the modified viral system for genome integration may eventually enable germline integration even at the pupal stage. Ando *et al.* (2007) injected worker larvae and pupae with a baculovirus system containing the necessary components for genome insertion; however, while they confirmed transient transgene expression, they were unable to confirm integration. The method would require further refinement to reduce mortality and improve efficiency to become a viable approach.

18.5.3   Local injection of CRISPR/Cas9 machinery

Sinakevitch *et al.* (2020) sought to test novel honey bee antibody specificity by knocking out the antibody targets (a GABA receptor subunit and a metabotropic glutamate receptor) in the honey bee brain. They achieved this by injecting the molecular machinery

Table 18.2. Promoters characterized for use in honey bees.

Promoter classification	Gene	Gene origin	Reference for usage in honey bees
Constitutive	*Am-actin5c*	Honey bee *actin5c* gene – housekeeping gene with strong constitutive expression	Schulte *et al.* (2013)
Universal species promoter	n/a	Artificial promoter	Schulte *et al.* (2014)
Viral	CMV	Human cytomegalovirus	Kunieda and Kubo (2004); Schulte *et al.* (2013)
Neuron-specific	*elp2l*	Homologue of *elav* in *Drosophila melanogaster*	Schulte *et al.* (2013)
Inducible (theoretically – not confirmed)	*Am-hsp70*	Honey bee gene with increased expression after heat shock	Schulte *et al.* (2013)
Inducible (theoretically – not confirmed)	*Dm-hsp70*	*Drosophila melanogaster* homologue to *Am-hsp70*	Robinson *et al.* (2000); Schulte *et al.* (2013)
Inducible (confirmed via cold-shock)	*Am-hsp83*	Honey bee *hsp83* gene – predicted to be induced by cold-shock based on homology	Schulte *et al.* (2013)

into the ocelli – small light-sensitive primitive 'eyes' located on the top of the honey bee's forehead. Direct injection into the brain, without performing dissections to expose the brain as needed for electroporation, is an attractive approach for localized gene editing. While it is an encouraging initial report, Sinakevitch *et al.* found that roughly half of the cells in the injection region were actually mutated and that the mutation efficiency will need to be improved. The authors included a detailed protocol in their publication, which should enable this technique to be easily reproduced and improved upon.

18.6 *piggyBac*- and CRISPR/Cas9-mediated Honey Bee Genome Editing by Embryonic Injection

Germline integration of a transgene was demonstrated in honey bees for the first time in 2014 using the *piggyBac* transposon system (Schulte *et al.*, 2014), but today, the CRISPR/Cas9 technique is more common; the high efficiency and flexibility of CRISPR/Cas9 editing has led to it being favoured: While transposable elements enable transgene insertion with little control over integration site, the CRISPR/Cas9 technique enables gene knockouts as well as site-specific knock-ins (Moon *et al.*, 2019) (see Ahmed and Wimmer, Chapter 5, this volume). Since 2016 there have been at least eight publications utilizing the CRISPR/Cas9 technique in honey bees (Kohno *et al.*, 2016; Kohno and Kubo, 2018; Hu *et al.*, 2019; Roth *et al.*, 2019; Değirmenci *et al.*, 2020; Chen *et al.*, 2021; Nie *et al.*, 2021; Wang *et al.*, 2021) and only one using the classic transposable element approach (Otte *et al.*, 2018). Still, these represent very few instances of honey bee gene editing in general; techniques for honey bee genetic engineering have been slow to develop and have advanced mainly due to the efforts of a handful of laboratories around the world. Despite the power of the technique, biological quirks of honey bee reproduction have made stable germline gene editing exceedingly difficult to achieve.

18.6.1 Improvements to the *piggyBac* transposon system

The *piggyBac* transposon system was originally developed from elements identified in baculoviruses and it has been commonly used in insect systems (Atkinson *et al.*, 2001) (see O'Brochta *et al.*, Chapter 1, this volume). Schulte *et al.* (2014) used this system to achieve the first stable germline integration of reporter genes into the honey bee genome; however, overall efficiency of the technique was low. Only 0.3–0.9% of injected eggs actually yielded queens carrying the transgene, partly due to low integration efficiency and partly due to queen loss at different stages of the rearing and screening process. Otte *et al.* (2018) therefore sought to improve the efficiency of the system and found that altering the injection location, the amount of transposase and the type of transposase improved transformation efficiency by over twofold (Fig. 18.2B) (Table 18.3). The researchers used a hyperactive transposase variant and adjusted its sequence to match typical honey bee codon usage, which contributed to transformation efficiency improvements even with large DNA cassettes (3.8–5.0 kb). So far, the *piggyBac* transposon system has only been utilized in proof-of-principle reports and not to investigate the biology of the honey bee, although at least one attempt has been made (McAfee, 2018). This is not to say that the technique is obsolete; gene insertion by transposable elements is still a suitable method for knocking in reporter-tagged genes, for example, especially when the specific integration site is not important.

18.6.2 CRISPR/Cas9 genome editing

The CRISPR/Cas9 genome editing system has already yielded substantial mechanistic insights into honey bee biology. Moreover, the technique is efficient enough that queens do not necessarily need to be produced; rather, worker eggs can be injected and reared to adulthood *in vitro*, avoiding animal losses during queen rearing and

Table 18.3. Improvements to *piggyBac*-based transformation rates. (Table adapted from Otte *et al*., 2018. CC-BY 4.0 (http://creativecommons.org/licenses/by/4.0/).)

	Conditions			
Transposase	hyTP	hyTP-Apis	hyTP-Apis	hyTP-Apis
Amount injected (pg)	90	90	240	240
Injection location	posterior	posterior	posterior	anterior
	Success rates			
% Queens with > 10% transgenic offspring	4%	11%	19%	25%
n	2/56	3/27	3/16	12/48

screening, and avoiding the need for an outdoor bee containment chamber to house the modified queens and their progeny. Three reports documented that a 100% mutation rate of injected embryos can be achieved using CRISPR/Cas9 (Hu *et al*., 2019; Roth *et al*., 2019; Wang *et al*., 2021), but in most cases 'mutation rate' refers to the frequency of injected embryos acquiring at least one mutated allele. A mutated individual may therefore have one non-functional gene copy and one wild-type copy, which could still result in a normal phenotype, depending on the trait being investigated. However, Roth *et al*. (2019) and Hu *et al*. (2019) found that up to 77% of mutated larvae had biallelic knockouts, which is a sufficient frequency to avoid needing to produce an interim queen to scale up mutant production. Other reports document lower frequencies of biallelic knockouts, from 36% to 49% (Değirmenci *et al*., 2020) and 53–72% (Chen *et al*., 2021), indicating that targeting different sequences, allelic variability of the bees and differences in laboratory protocols likely affects mutation efficiency.

Kohno *et al*. (2016) published the first example of using CRISPR/Cas9 in honey bees and demonstrated feasibility of the approach; since then, the technique has been used to produce mutant honey bees via embryonic injection on at least seven occasions, but only the most prominent are discussed here. Roth *et al*. (2019) used the CRISPR/Cas9 approach to investigate the sex-specific underpinnings of gonad development. The researchers knocked out *feminizer* (*fem*) and *doublesex* (*dsx*) genes in worker honey bees, which are alternatively spliced in female and male honey bees (Fig. 18.3A). They found that knocking out *fem* resulted in male gonad development, even though the injected embryos were genetically female, and knocking out *dsx* resulted in mostly female but some small male and intersex gonad development (Figs. 18.3B,C). Combined with their diet manipulations, they concluded that *fem* expression is necessary for diet-induced plasticity of gonad development. Wang *et al*. (2021) also investigated the sex-determination pathway by knocking out the *csd* gene, which resulted in the development of diploid males. In addition to documenting phenotypes associated with diploid males, which are seldom observed in nature and thus difficult to study, the researchers also performed transcriptomic profiling on the mutants to determine gene networks interacting with *csd*. Surprisingly, it is still a mystery how exactly *csd* drives such strong developmental differences in the heterozygous and hemi/homozygous states, and this is likely a topic that will be soon addressed via specific mutations using CRISPR/Cas9.

Insects in part rely on olfaction to communicate and pheromonal sensing has been an intense area of honey bee research. Now, researchers have used CRISPR/Cas9 to knock out *odorant receptor coreceptor* (*orco*) in honey bee workers in order to interrogate the gene's role in neural development. Chen *et al*. (2021) found that, unlike in fruit flies, *orco* is necessary for proper antennal lobe development. *Orco* knockout workers had fewer glomeruli in their antennal lobes, and glomeruli that did exist had altered morphology. Hundreds of antennal genes were also differentially expressed in knockout mutants
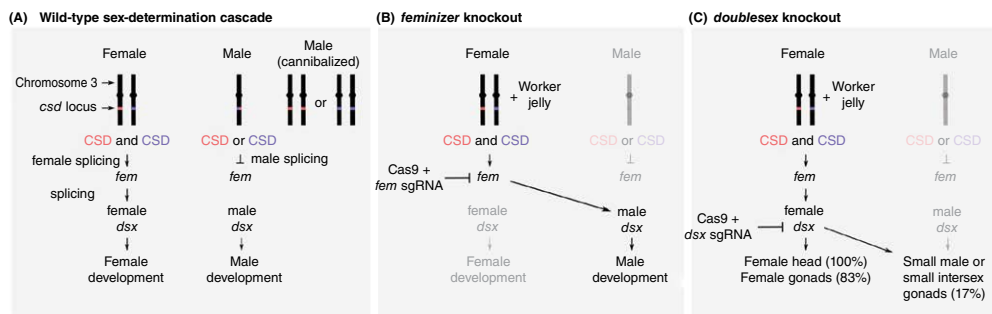
Fig. 18.3. Schematic of the honey bee sex-determination cascade and effects of CRISPR/Cas9-mediated knockout of components, as determined by Roth *et al*. (A) Overview of the wild-type cascade. CSD: complementary sex determination locus. Abbreviations: *fem*, *feminizer* gene; *dsx*, *doublesex* gene; sgRNA, single guide RNA. The heterozygous condition for the *csd* gene leads to female-specific splicing of *fem*, whereas the hemizygous or homozygous condition leads to male-specific splicing of *fem*, which renders the protein non-functional. **(B)** Effect of *feminizer* mutation. **(C)** Effect of *doublesex* mutation. (Figure originally published by McAfee *et al*. (2019) under a Creative Commons attribution license (CC BY 4.0).)

compared with controls, demonstrating that *orco* has far-reaching regulatory roles in the honey bee. These investigations into the roles of specific genes in sex determination and olfaction represent the kind of detailed mechanistic studies that have not been previously possible, and there are likely many more to come.

Excitingly, Nie *et al.* (2021) have demonstrated that knocking out a gene involved in cuticle pigmentation, *Amyellow-y*, yields a visually obvious phenotype of light-coloured cuticle. The researchers showed that a gene-edited queen will produce haploid progeny displaying the light-coloured phenotype, which allows germline gene editing to be easily identified without PCR screening. Therefore, it may be advantageous to include sg-RNA targeting *Amyellow-y* along with sg-RNA targeting an alternative gene of interest as an internal screening tool. Other work suggests that multiplexing gene targets within a single injection is feasible (Liu *et al.*, 2014; Li *et al.* 2017a,b) and the obvious phenotype of the *Amyellow-y* mutants may allow for simple, fast screening.

18.7   Industrial Applications

Once genetic mechanisms of economically desirable traits, such as pathogen or parasite resistance, tolerance to agrochemicals, or gentleness, have been identified, genetic engineering could theoretically become one avenue to produce bee stock carrying that trait (Grozinger and Zayed, 2020). There are currently many selective breeding projects around the world, mainly aimed at breeding honey bees that can resist the *Varroa destructor* parasite or brood diseases in order to reduce reliance on medications (Mondet *et al.*, 2020). The persistence of these breeding projects, despite the intensive resource and management cost involved, demonstrates that there is demand for resistant honey bee stock, and it is important to explore whether genetic engineering could help fill this demand or if the risk is deemed too great (see Hayes and Quinlan, Chapter 28, this volume).

18.7.1   Ethical considerations of commercial use

Depending on the specific trait and context, the risk of managing hives containing genetically engineered bees may or may not outweigh the potential reward and each proposal requires careful consideration. For example, making a genetic change that improves resistance to parasitic mites could be a more sustainable method of pest control than the current methods that rely on applying caustic

organic acid treatments to colonies or synthetic miticides that inevitably lead to the development of miticide resistance. If achievable, such a parasite-resistant engineered bee lineage would likely be in high demand; in fact, this is already the focus of several selective breeding programmes. Alternatively, introducing a genetic change that improves honey bee tolerance to agricultural pesticides might improve colony health during and after pollination; however, if honey bees are perceived as being protected from pesticides, this could promote pesticide over-application, to the detriment of native pollinators and other insects.

Each specific proposed genetic alteration needs to be subject to a carefully conducted risk assessment before any such strategy is developed for field use, while realizing that not every risk is possible to anticipate. Africanized honey bees, for example, are the unanticipated ultra-aggressive result of a cross between African honey bees and European honey bees, with the hybrid being significantly more aggressive than either of the progenitor lineages (Winston, 1992). With the technology available at the time, this outcome was not possible to predict, and is a pertinent example of unintended consequences when it comes to honey bee genetic manipulation. In addition to preliminary theoretical risk assessments, rigorous testing of the actual organisms must be conducted before potential commercial use can be considered; for example, field trials could be conducted on small islands where, if needed, complete extermination of colonies is possible.

Honey bee genome editing technology is not yet at the point to be used on a commercial scale, and regulatory frameworks for commercial use in many regions have not been established. Even for research use, regulations governing risk management of genetically engineered bees is surprisingly diverse. In some jurisdictions, it is sufficient to 'contain' genetically engineered queen bees outdoors using a simple queen excluder (metal or plastic bars of sufficient width to enable worker bees to pass, but not queens) fastened to the front of a hive, despite these not being 100% effective due to variation in queen size. In other locations, no genetically engineered bees are allowed outside strict containment facilities. Now is the time to openly debate the potential applications of honey bee genome editing and come to a consensus for regulation (see Beech *et al.*, Chapter 25, this volume).

18.7.2 Biological barriers to commercial viability

In addition to risk assessments and ethical considerations, there are also practical challenges limiting the utility of genetic engineering in industry. For example, as noted by Grozinger and Zayed, colony health is closely linked to genetic diversity of the colony (the queen's multiple-mating strategy leads to high within-colony diversity, despite all workers and drones being derived from the same mother) (Mattila and Seeley, 2007; Grozinger and Zayed, 2020). Single-drone inseminated queens can be produced for breeding programmes or for research, but they have poor productivity (Mattila *et al.*, 2012) and would therefore not be suitable for a commercial operation. The honey bee mating system would make it exceedingly difficult to produce a colony that is genetically diverse (the queen is inseminated with multiple genetically distinct drones) while all mating partners also carry the desired genetic change.

One way to circumvent this problem would be to utilize the CRISPR/Cas9 homing gene drive system in order to increase the frequency of homozygous mutations (Gantz and Bier, 2016) (see Raban and Akbari, Chapter 8; Champer, Chapter 9, this volume). If a queen carries a gene drive cassette in her germline, that genetic change has the capacity to copy itself into the wild-type chromosome when the egg becomes fertilized and a zygote begins to form. This approach would enable the production of a colony of bees that are homozygous for the desired mutation, but are still genetically diverse at all other loci. However, gene drives carry higher risk by their very nature: since they can copy themselves into wild-type chromosomes, they tend to amplify in the population rather than become diluted over

time (Webber *et al.*, 2015). Any application involving gene drives, regardless of the species, requires the highest scrutiny before it is allowed to exist passively in the environment.

The honey bee's mating strategy already presents a significant challenge for the maintenance of genetically edited populations or lineages, but it is not the only biological barrier. Many of the traits that would hold the greatest economic value are polygenic characteristics, like social immunity behaviours (such as hygienic behaviour, grooming behaviour, or varroa-sensitive hygiene) that lend resistance to parasitic mites or brood diseases (Evans *et al.*, 2006; Traynor *et al.*, 2020). While the traits that are most amenable to genetic manipulation are those that depend on a single allele with a well-characterized mechanism, polygenic traits are driven by multiple interacting genes and their underlying mechanisms are generally poorly understood in honey bees (Mondet *et al.*, 2020). Until these traits are better characterized, honey bee gene editing for any kind of commercial application will likely be limited to those with simpler genetic mechanisms.

18.8 Genome Editing of Honey Bee Symbionts

Honey bees live in partnership with a well-defined, stable community of gut bacteria which play important roles in the honey bee's immune activation, nutrient uptake, detoxification and development (Engel *et al.*, 2016; Kwong and Moran, 2016; Kwong *et al.*, 2017a; Wu *et al.*, 2020). This close interdependent relationship between the bee and its gut bacterial community makes it possible to alter the honey bee's biology by engineering the gut bacteria, rather than the bee itself (for examples of paratransgenesis approaches see Chapters 13–16, this volume). Although clearly not an example of honey bee genome editing *per se*, the technique is closely linked in theory and consequence. It also offers a more economically viable method for manipulating the biology of hosts and their pathogens, without actually genetically engineering either of them.

One innovative idea developed by Leonard *et al.* is to genetically engineer the honey bee's gut bacteria – specifically *Snodgrassella alvi*, one of the most abundant members of the gut community – to make honey bees more resistant to parasites and pathogens (Leonard *et al.*, 2018, 2020). Leonard *et al.* engineered *S. alvi* to produce a double-stranded RNA (dsRNA) sequence which targets essential genes in *Varroa destructor*, a devastating honey bee parasite, and deformed-wing virus (DWV), one of the most widespread honey bee viruses in the world. Although the mechanism has not been rigorously defined, researchers speculate that dsRNA produced in the gut is endocytosed by cells in the gut lining, and from there it can be dispersed to other tissues such as the haemolymph and fat body (Huvenne and Smagghe, 2010). When *V. destructor* feeds on the honey bee tissue, it in turn can ingest the dsRNA and is susceptible to its effects. Excitingly, Leonard *et al.* (2020) demonstrated that mites were more likely to die after feeding on honey bees inoculated with *S. alvi* engineered to produce varroa-lethal dsRNA, and likewise, bees were more likely to survive virus infection when inoculated with *S. alvi* engineered to produce DWV-targeting dsRNA sequences. They also demonstrated that host gene expression could be manipulated using this method.

Other researchers have demonstrated successful mutation of the honey bee gut parasite, *Lotmaria passim*, using CRISPR/Cas9 (Liu *et al.*, 2019). The purpose of the work was to develop tools for understanding host–pathogen interactions, such as how *L. passim* establishes infections in the gut. This type of basic host–pathogen research is sorely needed for honey bees. If similar techniques can be achieved for *Nosema* spp., which are intracellular fungal gut parasites that hijack host cell machinery in an approach similar to viruses, this could serve not only as a tool for studying pathogenesis, but also potentially for delivering genome editing machinery to host-gut epithelial cells.

Engineering gut bacteria and parasites to produce host- and pathogen-altering molecular machinery is an exciting, widely applicable technique, in part because engineering bacteria is much simpler than engineering honey

bees. Furthermore, inoculation of individual bees or colonies is a simple process of oral exposure. However, although the bees themselves do not receive a genetic change, risk consideration is still due. Some of the core honey bee gut microbes can colonize other species of bees (Kwong and Moran, 2016; Kwong *et al*., 2017b). Interspecific interactions can occur during robbing, predation, or shared forage resources, where there are opportunities to transmit modified bacteria to unintended species. Similar to risk assessments that are necessary for commercial use of engineered honey bees, assessments should be conducted prior to using modified bacteria in the field as well, especially since it could impact other host species.

18.9   Conclusion and Future Directions

The field of honey bee genome editing has been slow to develop but is now accelerating, in large part due to the continued improvement of the CRISPR/Cas9 gene editing system. Although the honey bee genome was sequenced as early as 2006 (Consortium, 2006), many of the genes it contains are still uncharacterized and gene expression manipulation techniques could be used to systematically decipher their functions (Elsik *et al*., 2018). Indeed, 'omic' technologies like proteomics and RNA-seq are only as powerful as our understanding of the original genome, and an improved knowledge of gene functions would likely necessitate reinterpretation of swaths of existing publicly available 'omic' data. Surprisingly fundamental mechanistic mysteries of honey bee biology still remain, such as exactly why the complementary sex determination locus functions so differently in the homozygous and heterozygous states to initiate alternate sex determination cascades. With the development of modern genome editing tools, the answers to such fundamental questions are imminent.

However, those tools still require further refinement. Despite improved transgene insertion and mutation efficiency, honey bee genome editing is still fundamentally limited by a lack of a high-throughput technique for introducing gene editing machinery into germ cells. Furthermore, although attempts have been made (Kitagishi *et al*., 2011; Goblirsch *et al*., 2013), there are no immortalized honey bee cell lines with which to screen constructs or single-guide RNA sequences – only primary cells or slow-growing embryonic cells (Goblirsch, 2017). Manual microinjection of thousands of eggs is necessary to produce a small number of modified individuals, which is prohibitively tedious for many research groups to perform. A high-throughput or less tedious delivery method, such as via sperm delivery (Robinson *et al*., 2000) or using the ReMOT technique (Chaverra-Rodriguez *et al*., 2018) (see Terradas *et al*., Chapter 6, this volume), would massively advance the field. Such technology is expected to be the next major breakthrough in honey bee gene editing methodology.

These methodological barriers, as well as biological barriers to strain propagation, mean that genome-edited honey bees are not likely to be produced for use in the beekeeping or agricultural industry soon. Even if these barriers are overcome, poor public perception and general distrust of gene editing technology would likely restrict such usage to only the most niche scenarios. However, the technique will continue to advance and innovations could change this trajectory. Now is the time for regulatory bodies to conduct risk assessments and implement regulatory frameworks to guide the potential introduction of this technology into the field. Until then, usage of gene editing technology will be restricted to helping us understand the basic biology of how these famous insect societies function.

References

Ando, T., Fujiyuki, T., Kawashima, T., Morioka, M., Kubo, T. and Fujiwara, H. (2007) *In vivo* gene transfer into the honeybee using a nucleopolyhedrovirus vector. *Biochemical and Biophysical Research Communications* 352, 335–340.

Atkinson, P.W., Pinkerton, A C. and O'Brochta, D.A. (2001) Genetic transformation systems in insects. *Annual Review of Entomology* 46, 317–346.

Brossard, D., Belluck, P., Gould, F. and Wirz, C.D. (2019) Promises and perils of gene drives: navigating the communication of complex, post-normal science. *Proceedings of the National Academy of Sciences USA* 116, 7692–7697.

Büchler, R., Andonov, S., Bienefeld, K., Costa, C., Hatjina, F. *et al.* (2013) Standard methods for rearing and selection of *Apis mellifera* queens. *Journal of Apicultural Research* 52, 1–30.

Chaverra-Rodriguez, D., Macias, V.M., Hughes, G.L., Pujhari, S., Suzuki, Y. *et al.* (2018) Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing. *Nature Communications* 9, 3008.

Chen, Z., Traniello, I.M., Rana, S., Cash-Ahmed, A.C., Sankey, A.L. *et al.* (2021) Neurodevelopmental and transcriptomic effects of CRISPR/Cas9-induced somatic orco mutation in honey bees. *Journal of Neurogenetics* 35, 320–332.

Consortium, H.G.S. (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* 443, 931–949.

Currie, R.W., Pernal, S.F. and Guzmán-Novoa, E. (2010) Honey bee colony losses in Canada. *Journal of Apicultural Research* 49, 104–106.

Dearden, P.K. (2006) Germ cell development in the honeybee (*Apis mellifera*); vasa and nanos expression. *BMC Developmental Biology* 6, 6.

Değirmenci, L., Geiger, D., Rogé Ferreira, F.L., Keller, A., Krischke, B. *et al.* (2020) CRISPR/Cas 9-mediated mutations as a new tool for studying taste in honeybees. *Chemical Senses* 45, 655–666.

Elsik, C.G., Tayal, A., Unni, D.R., Burns, G.W. and Hagen, D.E. (2018) Hymenoptera Genome Database: using HymenopteraMine to enhance genomic studies of hymenopteran insects. *Methods in Molecular Biology* 1757, 513–556.

Engel, P., Kwong, W.K., McFrederick, Q., Anderson, K.E., Barribeau, S.M. *et al.* (2016) The Bee Microbiome: impact on bee health and model for evolution and ecology of host-microbe interactions. *MBio* 7, e02164-15.

Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.L. *et al.* (2006) Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Molecular Biology* 15, 645–656.

Fleig, R. and Sander, K. (1986) Embryogenesis of the honeybee *Apis mellifera* L. (Hymenoptera: Apidae): an SEM study. *International Journal of Insect Morphology and Embryology* 15, 449–462.

Flenniken, M.L. and Andino, R. (2013) Non-specific dsRNA-mediated antiviral response in the honey bee. *PLoS ONE* 8, e77263.

Gantz, V.M. and Bier, E. (2016) The dawn of active genetics. *Bioessays* 38, 50–63.

Goblirsch, M. (2017) Using honey bee cell lines to improve honey bee health. In: Vreeland, R.H. and Sammataro, D. (eds) *Beekeeping – From Science to Practice*. Springer International Publishing, Cham.

Goblirsch, M.J., Spivak, M.S. and Kurtti, T.J. (2013) A cell line resource derived from honey bee (*Apis mellifera*) embryonic tissues. *PLoS ONE* 8, e69831.

Goulson, D., Nicholls, E., Botías, C. and Rotheray, E.L. (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* 347, 1255957.

Grozinger, C.M. and Zayed, A. (2020) Improving bee health through genomics. *Nature Reviews Genetics* 21, 277–291.

Hu, X.F., Zhang, B., Liao, C.H. and Zeng, Z.J. (2019) High-efficiency CRISPR/Cas9-mediated gene editing in honeybee. *G3: Genes, Genomes, Genetics* 9, 1759–1766.

Hung, K.J., Kingston, J.M., Albrecht, M., Holway, D.A. and Kohn, J.R. (2018) The worldwide importance of honey bees as pollinators in natural habitats. *Proceedings of the Royal Society B: Biological Sciences* 285, 20172140.

Huvenne, H. and Smagghe, G. (2010) Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. *Journal of Insect Physiology* 56, 227–235.

Ikeda, T., Nakamura, J., Furukawa, S., Chantawannakul, P., Sasaki, M. and Sasaki, T. (2011) Transduction of baculovirus vectors to queen honeybees, *Apis mellifera*. *Apidologie* 42, 461–471.

Jordan, A., Patch, H.M., Grozinger, C.M. and Khanna, V. (2021) Economic dependence and vulnerability of United States agricultural sector on insect-mediated pollination service. *Environmental Science and Technology* 55, 2243–2253.

Kitagishi, Y., Okumura, N., Yoshida, H., Nishimura, Y., Takahashi, J. and Matsuda, S. (2011) Long-term cultivation of in vitro *Apis mellifera* cells by gene transfer of human c-myc proto-oncogene. *In Vitro Cellular and Developmental Biology – Animal* 47, 451–453.

Klein, A.-M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A. *et al.* (2006) Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences* 274, 303–313.

Kohno, H. and Kubo, T. (2018) mKast is dispensable for normal development and sexual maturation of the male European honeybee. *Scientific Reports* 8, 11877.

Kohno, H., Suenami, S., Takeuchi, H., Sasaki, T. and Kubo, T. (2016) Production of knockout mutants by CRISPR/Cas9 in the European honeybee, *Apis mellifera* L. *Zoological Science* 33, 505-512.

Kucharski, R., Maleszka, J., Foret, S. and Maleszka, R. (2008) Nutritional control of reproductive status in honeybees via DNA methylation. *Science* 319, 1827–1830.

Kulhanek, K., Steinhauer, N., Rennich, K., Caron, D.M., Sagili, R.R. *et al.* (2017) A national survey of managed honey bee 2015–2016 annual colony losses in the USA. *Journal of Apicultural Research* 56, 328–340.

Kunieda, T. and Kubo, T. (2004) In vivo gene transfer into the adult honeybee brain by using electroporation. *Biochemical and Biophysical Research Communications* 318, 25–31.

Kwong, W.K. and Moran, N.A. (2016) Gut microbial communities of social bees. *Nature Reviews Microbiology* 14, 374–384.

Kwong, W.K., Mancenido, A.L. and Moran, N.A. (2017a) Immune system stimulation by the native gut microbiota of honey bees. *Royal Society Open Science* 4, 170003.

Kwong, W. K., Medina, L.A., Koch, H., Sing, K.W., Soh, E.J.Y. *et al.* (2017b) Dynamic microbiome evolution in social bees. *Science Advances* 3, e1600513.

Leonard, S.P., Perutka, J., Powell, J.E., Geng, P., Richhart, D.D. *et al.* (2018) Genetic engineering of bee gut microbiome bacteria with a toolkit for modular assembly of broad-host-range plasmids. *ACS Synthetic Biology* 7, 1279–1290.

Leonard, S.P., Powell, J.E., Perutka, J., Geng, P., Heckmann, L.C. *et al.* (2020) Engineered symbionts activate honey bee immunity and limit pathogens. *Science* 367, 573–576.

Li, M., Au, L.Y.C., Douglah, D., Chong, A., White, B.J. *et al.* (2017a) Generation of heritable germline mutations in the jewel wasp *Nasonia vitripennis* using CRISPR/Cas9. *Science Reports* 7, 901.

Li, M., Bui, M., Yang, T., Bowman, C.S., White, B.J. and Akbari, O.S. (2017b) Germline Cas9 expression yields highly efficient genome engineering in a major worldwide disease vector. *Proceedings of the National Academy of Sciences USA* 114, E10540–E10549.

Liu, Q., Lei, J. and Kadowaki, T. (2019) Gene disruption of honey bee trypanosomatid parasite. *Frontiers in Cellular and Infection Microbiology* 9, 126.

Liu, Y., Ma, S., Wang, X., Chang, J., Gao, J. *et al.* (2014) Highly efficient multiplex targeted mutagenesis and genomic structure variation in *Bombyx mori* cells using CRISPR/Cas9. *Insect Biochemistry and Molecular Biology* 49, 35–42.

Mansouri, M., Bellon-Echeverria, I., Rizk, A., Ehsaei, Z., Cianciolo Cosentino, C. *et al.* (2016) Highly efficient baculovirus-mediated multigene delivery in primary cells. *Nature Communications* 7, 11529.

Maori, E., Garbian, Y., Kunik, V., Mozes-Koch, R., Malka, O. *et al.* (2019) A transmissible RNA pathway in honey bees. *Cell Reports* 27, 1949–1959.e6.

Mattila, H.R. and Seeley, T.D. (2007) Genetic diversity in honey bee colonies enhances productivity and fitness. *Science* 317, 362–364.

Mattila, H.R., Reeve, H.K. and Smith, M.L. (2012) Promiscuous honey bee queens increase colony productivity by suppressing worker selfishness. *Current Biology* 22, 2027–2031.

McAfee, A. (2018) *Towards defining the molecular mechanism of hygienic behaviour in honey bees (Apis mellifera)*. PhD thesis. University of British Columbia, Vancouver.

McAfee, A., Chapman, A., Higo, H., Underwood, R., Milone, J. *et al.* (2020) Vulnerability of honey bee queens to heat-induced loss of fertility. *Nature Sustainability* 1–10, 367–376.

Mondet, F., Beaurepaire, A., McAfee, A., Locke, B., Alaux, C. *et al.* (2020) Honey bee survival mechanisms against the parasitic *Varroa destructor*: a systematic review of phenotypic and genotypic research efforts. *International Journal of Parasitology* 50, 433–447.

Moon, S.B., Kim, D.Y., Ko, J.H. and Kim, Y.S. (2019) Recent advances in the CRISPR genome editing tool set. *Experimental and Molecular Medicine* 51, 1–11.

Neumann, P. and Carreck, N.L. (2010) Honey bee colony losses. *Journal of Agricultural Research* 49, 1–6.

Nie, H.-Y., Liang, L.-Q., Li, Q.-F., Zhu, Y.-N., Guo, Y.-K. *et al.* (2021) CRISPR/Cas9 mediated knockout of Amyellow-y gene results in melanization defect of the cuticle in adult *Apis mellifera*. *Journal of Insect Physiology* 132, 104264.

Nunes, F.M., Aleixo, A.C., Barchuk, A.R., Bomtorin, A.D., Grozinger, C.M. and Simões, Z.L. (2013) Non-target effects of green fluorescent protein (GFP)-derived double-stranded RNA (dsRNA-GFP) used in honey bee RNA interference (RNAi) assays. *Insects* 4, 90–103.

Otte, M., Netschitailo, O., Kaftanoglu, O., Wang, Y., Page, R.E. and Beye, M. (2018) Improving genetic transformation rates in honeybees. *Science Reports* 8, 16534.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O. and Kunin, W.E. (2010) Global pollinator declines: trends, impacts and drivers. *Trends in Ecology & Evolution* 25, 345–353.

Potts, S.G., Imperatriz-Fonseca, V., Ngo, H.T., Aizen, M.A., Biesmeijer, J.C. *et al.* (2016) Safeguarding pollinators and their values to human well-being. *Nature* 540, 220–229.

Robinson, K., Ferguson, H., Cobey, S., Vaessin, H. and Smith, B.H. (2000) Sperm-mediated transformation of the honey bee, *Apis mellifera*. *Insect Molecular Biology* 9, 625–634.

Roth, A., Vleurinck, C., Netschitailo, O., Bauer, V., Otte, M. *et al.* (2019) A genetic switch for worker nutrition-mediated traits in honeybees. *PLOS Biology* 17, e3000171.

Schulte, C., Leboulle, G., Otte, M., Grünewald, B., Gehne, N. and Beye, M. (2013) Honey bee promoter sequences for targeted gene expression. *Insect Molecular Biology* 22, 399–410.

Schulte, C., Theilenberg, E., Müller-Borg, M., Gempe, T. and Beye, M. (2014) Highly efficient integration and expression of *piggyBac*-derived cassettes in the honeybee (*Apis mellifera*). *Proceedings of the National Academy of Sciences USA* 111, 9003–9008.

Sinakevitch, I., Kurtzman, Z., Choi, H.G., Ruiz Pardo, D.A., Dahan, R.A. *et al.* (2020) Anti-RDL and anti-mGlutR1 receptors antibody testing in honeybee brain sections using CRISPR-Cas9. *Journal of Visualized Experiments* 155, e59993.

Smith, K.E., Weis, D., Amini, M., Shiel, A.E., Lai, V.W.-M. and Gordon, K. (2019) Honey as a biomonitor for a changing world. *Nature Sustainability* 2, 223.

Snodgrass, R.E. (1910) *The Anatomy of a Honey Bee*. Government printing office, Bureau of Entomology, Washington, DC.

Traynor, K.S., Mondet, F., De Miranda, J.R., Techer, M., Kowallik, V. *et al.* (2020) *Varroa destructor*: a complex parasite, crippling honey bees worldwide. *Trends in Parasitology* 36, 592–606.

Wang, X., Lin, Y., Liang, L., Geng, H., Zhang, M. *et al.* (2021) Transcriptional profiles of diploid mutant *Apis mellifera* embryos after knockout of csd by CRISPR/Cas9. *Insects* 12, 704.

Webber, B.L., Raghu, S. and Edwards, O.R. (2015) Opinion: Is CRISPR-based gene drive a biocontrol silver bullet or global conservation threat? *Proceedings of the National Academy of Sciences* 112, 10565–10567.

Winston, M.L. (1991) *The Biology of the Honey Bee*. Harvard University Press, Cambridge, Massachusetts.

Winston, M.L. (1992) The biology and management of Africanized honey bees. *Annual Review of Entomology* 37, 173–193.

Wu, Y., Zheng, Y., Chen, Y., Wang, S., Hu, F. and Zheng, H. (2020) Honey bee *(Apis mellifera)* gut microbiota promotes host endogenous detoxification capability via regulation of P450 gene expression in the digestive tract. *Microbial Biotechnology* 13, 1201–1212.

Xu, J., Xu, X., Zhan, S. and Huang, Y. (2019) Genome editing in insects: current status and challenges. *National Science Review* 6, 399–401.

Yusa, K., Zhou, L.Q., Li, M.A., Bradley, A. and Craig, N.L. (2011) A hyperactive *piggyBac* transposase for mammalian applications. *Proceedings of the National Academy of Sciences*. USA 108, 1531–1536.

19 Progress Towards Germline Transformation of Ticks

Michael Pham[1], Arvind Sharma[1], Andrew Nuss[2] and Monika Gulia-Nuss[1]*

[1]*Department of Biochemistry and Molecular Biology, University of Nevada, Reno, USA;* [2]*Department of Agriculture, Veterinary, and Rangeland Sciences, University of Nevada, Reno, USA*

19.1 Introduction

Ticks are vectors of medical and veterinary importance and can transmit a variety of pathogens, including viruses, bacteria, protozoans and fungi (Jongejan and Uilenberg, 2004). Pathogens are either taken up during a bloodmeal, carried over transstadially, or in some cases transovarially (such as rickettsiae and flaviviruses) or even mechanically transmitted when tick feeding is interrupted (Lubinga *et al*., 2015). Ticks and the diseases they transmit incur great costs to public health and agriculture across the world (Jongejan and Uilenberg, 2004). For instance, *Ixodes scapularis*, the major vector of Lyme disease in the USA, alone is responsible for over 300,000 cases of Lyme disease annually (Centers for Disease Control and Prevention, 2015), in addition to other pathogens, including species of *Anaplasma*, *Babesia*, *Bartonella*, *Ehrlichia*, *Rickettsia*, *Theileria* and *Flavivirus* (Nelder *et al*., 2016). Ticks also impact livestock production. *Rhiphicephalus* (*Boophilus*) *microplus*, for instance, is a one-host tick harmful to cattle and has spread from its origin in Southeast Asia to near-worldwide distribution (Jongejan and Uilenberg, 2004). *R. microplus* is a vector for *Babesia* and *Anaplasma* pathogens and can incur significant agricultural costs, even in cases without pathogen transmission (Jongejan and Uilenberg, 2004). The economic losses due to *R. microplus* can be expressed in terms of either reduced body weight and milk production or treatment cost employed for prevention of disease and control of ticks. Controlling *R. microplus* and other ticks requires the use of acaricides, leading to multi-acaricide resistance (Jongejan and Uilenberg, 2004).

The study of tick biology and host–pathogen interactions has been severely limited due to the lack of genetic tools, with the exception of RNA interference (RNAi)-mediated knockdown, to study gene function (Kocan *et al*., 2011; de La Fuente, 2021) (see De Schutter and Smagghe, Chapter 4, this volume, for background on RNAi). However, RNAi has limitations, including variable knockdown of targets and a transient timeline. This primary dependence on RNAi, and the absence of other genetic tools, stems from the lack of an efficient genetic transformation protocol. Our preliminary work has permitted us to overcome this impediment through the development of embryo and adult injection protocols.

Effective embryo injections require knowledge of early embryological events, principally

*Corresponding author, email: mgulianuss@unr.edu

the timing of cellularization, and potentially localizing germline cells within the egg. This is important so that introduced material can access the nucleus (prior to cellularization) and create stable germline transformants. Ideally, microinjection needs to occur before cellularization, but not too early as premature injection can cause significant lethality (Eggleston and Meredith, 2014) (see O'Brochta, Chapter 1, this volume).

No heritable insertions have been observed in ticks yet. Successful integration of DNA into the tick genome requires molecular tools such as transposable genetic elements, recombinases, or CRISPR/Cas9. Additionally, functional promoters are needed to drive the expression of transgenes to study gene function and reporter genes as markers of transgenic individuals. For the intent of this chapter, we focus on current progress in tick embryology, functional promoters and reporter systems in tick cell lines, and delivery methods for genetic transformation.

19.2   Tick Embryogenesis

The female genital system in Ixodidae consists of a single tubular U-shaped ovary in the posterior region of the body, oviducts (paired, coiled, or folded), a uterus, accessory glands, and a vagina and genital aperture (Brinton and Oliver, 1971). Tick ovaries are panoistic and the oocytes attach to the ovarian wall by pedicel cells that face the haemocoel before deposition into the ovarian lumen. The pedicel cells have been proposed as being analogous to the follicle and nurse cells of insect ovaries (de Oliveira *et al*., 2007). The glandular Gené's organ, a paired organ found only in ticks (Arthur, 1953), is remote from the genital tract but important for egg survival because of the finger-like extensions (horns) that protrude through the camerostomal cavity and wax the eggs during egg laying, providing desiccation protection (Booth, 1989; Kakuda *et al*., 1994; Ogihara and Taylor, 2014). Tick eggs are spherical to ovoid-oblong. The surface is smooth and glossy due to the wax layer and lacks any reticulation. Newly

deposited eggs are pale yellow and become deep rusty brown as embryonic development proceeds. No orientation (dorsal, ventral, or lateral) is readily evident in the early stages of embryo development.

The key early embryonic events such as nuclear division, timing of cellularization, gonadal cell formation and developmental timing are important to understand for generating successful, heritable transgenic organisms. While considerable information on embryonic development in the Acarina is available, most studies are on the embryonic development of mites (Laumann *et al*., 2010; Chetverikov and Desnitskiy, 2016). Information on tick early embryonic development remains scarce and several aspects, as described below, remain to be confirmed. Hard tick life cycles vary depending on their life histories: one-host, two-host, or three-host ticks, based on the number of vertebrate hosts required to complete the life cycle. While embryonic development in one-host ticks is relatively short (11–21 days for most tick species), it may take up to 35–45 days in three-host ticks. Therefore, key events also occur on different time scales in different tick species. Except for an embryonic development study on *Ixodes calcaratus* (Wagner, 1894), published over a century ago in Russian, no other description of the embryonic development of any member of the genus *Ixodes* appears in the literature. Similarly, we could not find any studies on early embryogenesis in *Amblyomma* ticks. However, studies in other genera, such as *Dermacentor* (Pressesky, 1952; Friesen *et al*., 2016) and *Rhiphicephalus* (*Boophilus*) (Campos *et al*., 2006; Santos *et al*., 2013), have been conducted in detail. In this section, we review and compare early embryonic development in the ixodid (hard) and argasid (soft) ticks (Fig. 19.1).

19.2.1   Early embryonic development in *Dermacentor* spp.: *D. andersoni* and *D. variabilis*

Commonly known as the Rocky Mountain wood tick, *Dermacentor andersoni* is a three-host
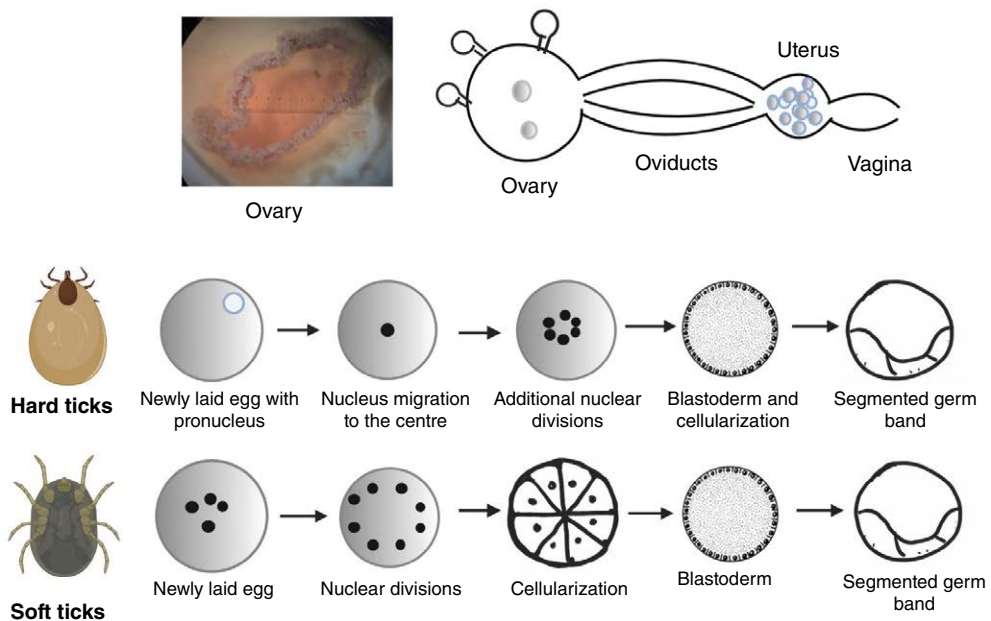
Fig. 19.1. Graphical illustration of early embryonic development in ixodid (hard) and argasid (soft) ticks. Except for *Dermacentor variabilis*, most studies in the hard ticks suggest that fertilization occurs after egg laying. Early divisions appear to occur without cellularization and a syncytium is predicted to form. In contrast, in the only species of soft ticks studied so far, embryos appear to be fertilized with a few early mitotic divisions at the time of egg laying. Cellularization appears to occur at the 8-cell stage and possibly even before that time point. Reproductive tract diagram is modified from Mehlhorn *et al*., 2016. Created by Biorender.com

tick. The complete life cycle requires 2 years to complete in the field. The embryonic development period is approximately 6 weeks at 20–22°C (Presseky, 1952) but could be shortened to about 18 days at 25°C and 93% relative humidity (RH) (Friesen *et al*., 2016). Pressesky (1952) provided a full account of embryonic development starting from eggs as young as < 1 h after egg laying (AEL), one of the few studies to date utilizing young embryos. Eggs < 1 h old had a visible female pronucleus at the periphery of the egg, suggesting that fertilization takes place following oviposition. Fertilization after egg deposition was also observed in *Ixodes calcaratus* (reviewed by Pressesky, 1952). Fertilization in *D. andersoni* eggs was not observed; however, syngamy was thought to occur sometime before 4 h AEL as suggested by the appearance of a synnucleus and three polar bodies. The nucleus then migrated to the centre of the egg, where the first mitotic division took place at approximately 12 h

AEL. The synchronous nuclear division continued and endoderm cells appeared at approximately 8 days AEL in the region of the future caudal lobe of the germ band. More recently, Friesen *et al*. (2016) also suggested few nuclear divisions by 24 h AEL. However, *D. andersoni* eggs in their experiments hatched within 18 days instead of 6 weeks, complicating timeline comparisons. At 24 h AEL, the embryos were already near the fifth mitotic division and the nuclei were located at the periphery of the egg (Friesen *et al*., 2016). Based on these early divisions, Friesen *et al*. (2016) suggested that if nuclear division occurs at a constant rate, the post-oviposition mitotic division rate in *D. andersoni* will be every 5 h. This nuclear division rate is much slower than described in *Drosophila melanogaster*, where early mitotic divisions occur as fast as every 8 min (Foe and Alberts, 1983; Gilbert, 2000).

Pressesky (1952) suggested that blastoderm formation is complete at about 4–5

days AEL whereas it was complete within 2–3 days in the work by Friesen *et al.* (2016). This discrepancy is most likely due to differences in rearing conditions. At 2–3 days AEL, Friesen *et al.* observed the formation of a distinct cell cluster similar to the cumulus of spiders (Akiyama-Oda and Oda, 2003). Migration of this cluster to one pole appeared to give rise to the germ band which marked the establishment of a dorsal–ventral body axis. Neither of these studies observed cell membrane formation.

Commonly known as the American dog tick, *Dermacentor variabilis* is a three-host tick. The life cycle requires a minimum of 54 days to complete but can take up to 2 years, depending on host availability and temperature (Matheson, 1950; Campbell, 1979). While in *D. andersoni* fertilization has been reported after oviposition, early embryonic development appears to differ greatly in *D. variabilis*. The only report on this species is from the early 1900s. Zebrowski (1926) suggested that fertilization occurs in oviducts because in gravid females the entire reproductive tract (ovaries, oviduct and uterus) was filled with eggs in the blastoderm stage. The presence of sperm along the reproductive tract in non-gravid females suggested that the eggs fertilize before reaching the uterus. This difference in early embryo development could be attributed to the relatively shorter life cycle and therefore shorter embryonic development time in *D. variabilis*.

19.2.2 Early embryonic development in *Rhiphicephalus* (*Boophilus*) *microplus*

Commonly known as the cattle tick, *Rhiphicephalus microplus* is a one-host tick. Larval, nymphal and adult development of this tick takes place on a single bovine host within 2–4 weeks. The embryonic development period is approximately 21 days (Campos *et al.*, 2006). Santos *et al.* (2013) developed the first tick embryo staging system in this species based on spider embryogenesis (McGregor *et al.*, 2008; Wolff and Hibrant, 2011; Mittmann and Wolff, 2012). However, the youngest embryos used for staging

were 24 h AEL and early development in this genus has yet to be described. The authors used pTyr antibody staining to visualize cell membrane formation and concluded that the cellularization occurs early in tick development (within 24 h AEL in this species). However, Campos *et al.* (2006), using similar rearing conditions, suggested that until 5 days AEL, the *R. microplus* embryo is a syncytium (a single cell containing several nuclei, formed by nuclear division) and cellularization occurs at 6 days AEL, followed by a segmented germband on day 7 AEL. Because of these conflicting results, early nuclear and cellular divisions need to be confirmed in *R. microplus*.

19.2.3 Early embryonic development in *Ixodes* spp.: *I. calcaratus* and *I. scapularis*

Early embryonic division in *I. calcaratus*, a three-host tick native to northern Asia, was studied in detail over a century ago (Wagner, 1894). Although this article is not readily available, Pressesky (1952) provided an excellent summary of the observations. According to this summary, *I. calcaratus* embryogenesis is similar to *D. andersoni*. Wagner suggested that syngamy occurred after egg laying at the periphery of the egg and that the synnucleus moved to the central part of the egg at approximately 5 h AEL. Our unpublished results in the black-legged tick, *I. scapularis*, also agree with embryogenesis in *I. calcaratus*. No nucleus was visible in eggs at 0–5 h AEL and a single nucleus appeared in the centre of the egg at 6 h AEL (unpublished data). No other studies have examined early embryogenesis in *Ixodes* spp.

19.2.4 Early embryonic development in *Hyalomma dromedarii*

The embryology of the camel tick, *Hyalomma dromedarii*, a two-host tick distributed mainly in North Africa, is described from a single study of serial sections of softened chorion eggs, starting from the newly laid

eggs (El-Kammah *et al.*, 1982). Based on the vitellophages of different sizes, the authors suggested that the cleavage began within 3 h AEL. Several mitotic divisions forming a spherical mass were observed 6–30 h AEL. Nuclei migrated to the periphery by 30 h AEL. Mitotic division and nuclei migration continued for 8 days until blastoderm formation followed by the germ band formation at 10 days AEL (El-Kammah *et al.*, 1982).

19.2.5 Early embryonic development in an argasid (soft) tick, *Ornithodorus moubata*

Commonly known as the African hut tampan or the eyeless tampan, *Ornithodorous moubata* is native to Africa. Embryonic development of *O. moubata* was described by Aeschlimann (1958). This study described superficial cleavage and suggested that the egg preserves a syncytial organization as the dividing nuclei migrate towards the yolk surface. After eight mitotic cycles the cleavage nuclei reach the periphery and subsequently cellularization occurs, leading to the formation of the superficial blastoderm. Reinvestigation of *O. moubata* embryogenesis confirmed a total cleavage starting from the eight-cell stage and suggested that perhaps cleavage is total from the beginning. However, eggs prior to the eight-cell stage were not observed (Fagotto *et al.*, 1988).

19.2.6 Tick embryology conclusions

In summary, several researchers consider total cleavage to be the rule during embryogenesis among the Ixodidae, which is mainly based on one ultrastructural study of the soft tick, *O. moubata*. Whether or not the early divisions in tick embryos are holoblastic (with mitosis and cytokinesis) or syncytium (nuclear division only) remains to be confirmed. Further investigations into the early embryology of Acari will contribute to a re-examination of the interpretations of chelicerate development and will potentially settle the debate over total cleavage versus

syncytium formation. These investigations will also provide the necessary information on suitable timing for embryo injection for efficient transgenic tick development.

19.3 Transformation Markers and Promoters

The ability to screen for transgenic individuals remains a challenge in non-model organisms. Two methods are commonly used: (i) a visible physical marker such as the *white* eye-colour gene from *D. melanogaster*; and (ii) a fluorescent marker such as enhanced green fluorescent protein (EGFP) under the control of a functional promoter (see O'Brochta, Chapter 1, this volume).

19.3.1 Potential physical markers

A physical phenotype visible to the eye is highly desirable for transgenesis screening. One possible phenotype to target in ticks is cuticle colour. For instance, a protein involved in melanin biosynthesis, the dopachrome converting enzyme/yellow protein (DCE), has been successfully targeted for mosquito transgenesis, resulting in a yellow cuticle phenotype when disrupted (Li *et al.*, 2017). We identified a putative DCE orthologue in the *I. scapularis* genome (ISCW009232) (Nuss *et al.*, 2021) that may have a similar function in ticks. Another potential gene related to pigmentation is putative pink-eyed dilution protein, P-protein (ISCW005393). The human orthologue of P-protein is involved in the normal colouring of skin, eyes and hair and is likely involved in melanin production (Brilliant, 2001).

Morphological phenotypes (such as extra legs and mouthpart abnormalities), often associated with homeobox genes in arthropods, are also easy to screen but may incur survival costs. Published reports of tick abnormalities include abnormal mouthparts (Sharma *et al.*, 2020), extra legs in adult and nymphal stage ticks (Larson and Paskewitz, 2016) and dwarfism (Soghigian

et al., 2017). However, except for our work in review (Sharma *et al.*, 2020), there is currently no data connecting the phenotypes to genetic mutations or loci. Therefore, additional work is needed to identify genes associated with these phenotypes.

19.3.2 Promoters

Protocols for generating transgenic ticks require functional promoters that are either ubiquitous, tissue-specific, or inducible (see Schetelig *et al.*, Chapter 2; Nolan and Hammond, Chapter 3, this volume). Promoters derived from distant species may not always express consistently or even be functional (Schetelig and Handler, 2013; Matthews and Vosshall, 2020). Often the most effective promoters for transgene expression, particularly tissue-specific promoters, are specific to the species (Moreira *et al.*, 2000; Kusakisako *et al.*, 2018). However, in organisms with large, repetitive genomes, like ticks (Gulia-Nuss *et al.*, 2016; Jia *et al.*, 2020), identifying promoter or enhancer sequences remains challenging (Matthews and Vosshall, 2020). This highlights the need to identify both endogenous and non-endogenous promoters that may function across multiple tick species. Identification of effective and consistent promoter or enhancer sequences – endogenous or non-endogenous – for use in driving transgene expression is an important step for the development of tick transgenesis.

In this section, we describe characterized tick endogenous promoters as well as non-endogenous promoters. Except for one promoter (*polyhedrin*), all of the promoters described have only been verified in tick cell culture models (Table 19.1).

19.3.3 Endogenous tick promoters

Subolesin promoter

Subolesin was initially discovered in an immunization screen using a cDNA library from an *I. scapularis* cell line (IDE8), derived from tick embryos (Almazán *et al.*, 2003). It was subsequently identified as an orthologue to *akirin* in insects and vertebrates (Galindo *et al.*, 2009), a protein involved in the innate immune pathway. The *subolesin* promoter cloned from the *I. scapularis* cell line, ISE6, was used to identify the 356 bp core promoter that is regulated by NF-KB (Relish) (Naranjo *et al.*, 2013). Sequence analysis of the promoter revealed a putative NF-KB binding site (GTGTCTTTCC) with 80% identity to the consensus sequence. Chemical induction of NF-KB activity with 2-deoxy-D-glucose or repression by sodium salicylate also induced or repressed *subolesin* transcript and protein levels in ISE6 cells (Naranjo *et al.*, 2013). Together these data suggest that the *subolesin* promoter is activated by NF-KB.

The function and regulation of the *subolesin* gene, including its promoter, remains unclear. Gene expression data indicate subolesin expression in salivary gland, midgut, ovary and integument (Liu *et al.*, 2016; Artigas-Jerónimo *et al.*, 2018) and multiple life stages (Artigas-Jerónimo *et al.*, 2018), but there may be tick-specific differences in expression in response to stimuli (Liu *et al.*, 2016). Overall, this promoter shows good potential for screening for presence of transformation and perhaps for refractory genes. However, due to the variability in response to pathogen infection and its multifunctional role (Artigas-Jerónimo *et al.*, 2018), it may not be a good promoter to use in response to infection or for gene function studies.

Ferritin promoter

Ferritin is a 24-subunit, near-universal protein (found in most eubacteria, archaea, plants and animals, except yeast) that stores iron in a non-toxic form (Arosio *et al.*, 2009). Ferritin is controlled transcriptionally and post-transcriptionally by several factors, including labile iron through interactions between iron response element (IRE) sequences and iron regulatory proteins (IRP). Ferritin is especially important for haematophagous arthropods, as they must either metabolize or sequester large amounts of toxic iron from

Table 19.1. (a) Endogenous and (b) non-endogenous functional promoters in ticks.
(a) Endogenous promoters

Gene promoter (species)	Size (bp)	Functional context	Tissue	Reference
Subolesin (*I. scapularis*)	356	IDE8 (*I. scapularis*)	Salivary gland (SG) and midgut (MG)	Naranjo *et al.*, 2013
Ferritin (*H. longicornis*)	639	ISE6 (*I. scapularis*)	SG, MG, ovaries (OV)	Hernandez *et al.*, 2019
Actin (*H. longicornis*)	1373	ISE6	All (predicted)	Kusakisako *et al.*, 2018
Ribosomal protein L4 - rpl4 (*R. microplus*)	674	BME26 (*R. microplus*)	All (predicted)	Tuckow and Temeyer, 2015
Elongation factor 1α – EF-1α	893	BME26	All (predicted)	ibid.
CZEst9 - Pbm	1029	BME/CVTM (*R. microplus*)	No tissue expression data	Machado-Ferreira *et al.*, 2015

(b) Non-endogenous promoters

Gene promoter (species)	Size (bp)	Functional context	Tissue	Reference
AcMNPV *Polyhedrin*	92	*H. longicornis* adults and larvae	SG, potentially other tissue	You *et al.*, 2003
Phosphoglycerate kinase (human)	500	BME26, ISE6	All (predicted)	Kusakisako *et al.*, 2018; Tuckow and Temeyer, 2015
CAG	1675	BME26, ISE6	All (predicted)	Naranjo *et al.*, 2013; Esteves *et al.*, 2008; Kurtti *et al.*, 2008
CMV-IE	508			ibid.
SV40	317			Naranjo *et al.*, 2013
CAM35S (Cauliflower Mosaic Virus)	343	BME/CVTM	All (predicted)	Machado-Ferreira *et al.*, 2015

the bloodmeal (Xu *et al.*, 2004). The amount of iron ingested in the bloodmeal makes ferritin expression potentially bloodmeal inducible. Since the majority of tick-borne pathogens are acquired through an infected bloodmeal, a bloodmeal-inducible promoter to express refractory transgenes is expected to be active during the initial stages of pathogen infection and dissemination from the midgut tissue (Moreira *et al.*, 2002; Hernandez *et al.*, 2019).

The *ferritin1* gene is expressed ubiquitously in *Haemaphysalis longicornis* ticks and has increased transcript levels after a bloodmeal (Galay *et al.*, 2013). Initial testing using dual luciferase constructs demonstrated that the *H. longicornis ferritin1* (*HlFer1*) putative promoter (2906 bp) had lower luciferase activity than the promoter-less control (Kusakisako *et al.*, 2018). The authors

suggested that the lower activity could be due to the *HlFer1* promoter region containing a suppression sequence, supported by prior published work showing the absence of *HlFer1* protein in the unfed whole larva, nymph, or adult *H. longicornis* extracts, despite the presence of *HlFer1* transcript (Galay *et al.*, 2013). In a follow-up study, Hernandez *et al.* (2019) further truncated the *HlFer1* promoter region to a core promoter. This core region had maximal induction to 1 mM ferrous sulfate to release IRP suppression in ISE6 cells.

Galay *et al.* (2013) reported that a bloodmeal induced higher HlFer1 protein levels in salivary glands and midgut tissue. In contrast, in the same study, HlFer1 protein was present in ovaries in unfed ticks but was not detected in partially engorged, fully fed and post-engorgement timepoints

(Galay *et al.*, 2013). This suggests different regulatory mechanisms either post-transcriptionally or during protein translation in ovaries for HlFer1. It remains to be seen whether this unique regulation in ovary tissue would apply to the *HlFer1* core promoter characterized by Hernandez *et al.* (2019).

Eight additional tick *ferritin* gene sequences from different tick species have been described (Xu *et al.*, 2004). IRE sequences (27 bp) in these genes were highly conserved (1 bp change in *I. scapularis*, and 2 bp differences in the soft tick, *O. moubata*). These data suggest conserved regulation of *ferritin1* transcription in ticks that may allow cross-tick species function from a tick-derived ferritin promoter. Together these lines of evidence suggest that *HlFer1* could be used as a bloodmeal-inducible promoter targeting multiple tissues with cross-tick species functionality.

Actin promoter

The 1373 bp putative promoter for an *actin* gene in *H. longicornis* (*HlActin*) was cloned into a luciferase reporter plasmid with confirmed activity (Kusakisako *et al.*, 2018). The *HlActin* promoter was compared with the synthetic CAG and human *phosphoglycerate kinase* (*PGK*) promoters, both confirmed to work in BME26 and ISE6 cells (Kusakisako *et al.*, 2018). The *HlActin* promoter had almost the same activity as the *PGK* promoter, but less than the CAG promoter (Kusakisako *et al.*, 2018). The *HlActin* promoter had strong transcriptional activity in *I. scapularis* cells and it is likely that this promoter could work in both *I. scapularis* and *H. longicornis* and is likely functional in other tick species and cell lines.

Ribosomal protein L4 and elongation factor 1α

A 674 bp promoter fragment for the *ribosomal protein L4* (*rpl4*) gene from *R. microplus* was identified as the most active in a promoter screen conducted in BME26 (*R. microplus*) cells driving luciferase (Tuckow and Temeyer, 2015). An 893 bp promoter fragment from the *elongation factor 1α* (*EF1α*)

gene was also active but had approximately 5% of the luciferase activity of the *rpl4* and was not further examined. A relevant point to note is that Tuckow and Temeyer were unable to demonstrate *Renilla* luciferase activity in the BME26 line. This may mean that *Renilla* luciferase does not work in certain tick cell lines, although *Renilla* luciferase is active in ISE6 cells (personal communication, Prof. Timothy Kurtti, University of Minnesota). However, firefly, Cypridina and NanoLuc® luciferases did show activity in BME26 cells (Tuckow and Temeyer, 2015), suggesting that different assay platforms might be needed for confirmation of promoter activity in specific tick cell lines.

Pyrethroid-metabolizing esterase gene CzEst9 (PBm) promoter

The *R. microplus CzEst9* gene encodes a pyrethroid-metabolizing esterase (Guerrero and Nene, 2008). EGFP–EGFP and Salp15 (*I. scapularis*)–EGFP fusion proteins were expressed in BME/CVTM (*R. microplus*) cells under the *CzEst9* 1029 bp promoter by Machado-Ferreira *et al.* (2015) using *Agrobacterium tumefasciens*-mediated gene transfer. EGFP was detected in BME/CVTM cells by microscopy, and EGFP and Salp15 were detected by immunoblot in BME/CVTM cell lysate (Machado-Ferreira *et al.*, 2015). Currently, there is no additional data on the core promoter and tissue- or stage-specific expression of the *CzEst9* gene.

19.3.4   Non-endogenous promoters in ticks

The advantage of using non-endogenous promoters for transgene expression is that these promoters are potentially functional across multiple species, saving time in developing and testing endogenous promoter constructs for each tick species. For instance, the *3xP3* synthetic promoter composed of three Pax6 binding sites and minimal *hsp70* promoter from *D. melanogaster* is functional in several different insect species (Berghammer *et al.*, 1999; Schetelig and Handler,

2013). In this section, we discuss six non-endogenous promoters that have been tested in tick cells.

Polyhedrin promoter from Autographa californica multiple nuclear polyhedrosis virus (AcMNPV)

The AcMNPV baculovirus has been used to drive the expression of hundreds of proteins in insect cells using either the *p10* gene (10 KDa protein) or the *polyhedrin* gene promoter, usually active approximately 48 h after viral infection (Ramachandran *et al.*, 2001). The *polyhedrin* gene in the AcMNPV genome encodes for a protein that creates a crystalline matrix that protects the fragile virus particle from environmental elements (Ramachandran *et al.*, 2001). However, under laboratory conditions and cell culture, the polyhedrin matrix is not necessary, so the *polyhedrin* gene can be replaced with a protein encoding a gene of choice. The *polyhedrin* promoter is considered a strong promoter capable of driving recombinant protein expression to 25–50% of total cellular protein content (Ramachandran *et al.*, 2001).

V5- and his-tagged chitinase under the AcMNPV *polyhedrin* promoter was expressed in tick salivary glands and whole larvae of *H. longicornis* (You *et al.*, 2003). The authors used an insect cell line, Sf9, supernatant containing recombinant AcMNPV (pBlueBac4.5/V5-His transfer vector, Invitrogen). This supernatant was applied topically to the dorsal side of *H. longicornis* larvae and adults. V5-tagged chitinase was detected in salivary glands (adults) and whole larvae by western blotting (You *et al.*, 2003). To our knowledge, this is the only published report of recombinant protein expression in tick tissue in live ticks.

Human phosphoglycerate kinase promoter

The human *phosphoglycerate kinase* (*PGK*) gene promoter is part of the pmirGLO dual reporter vector that was used to investigate the *R. microplus rpl4* and *EF-1α* gene promoters, noted above (Tuckow and Temeyer, 2015). In fact, the *PGK* promoter induced the highest luciferase activity among all promoters tested in that study, with detectable activity in 2 days post-transformation and maximal activity at 5 days post-transformation in BME26 tick cells (Tuckow and Temeyer, 2015). Curiously, the human *PGK* promoter shows weak activity in six mammalian cell lines including human cells (Qin *et al.*, 2010) but appears to have strong transcriptional activity in two *Drosophila* cell lines (Qin *et al.*, 2010) and at least two tick cell lines (BME26 and ISE6 cells) (Tuckow and Temeyer, 2015; Kusakisako *et al.*, 2018).

CAG, cytomegalovirus immediate early, and Simian Virus 40 promoters

The *CAG* promoter was synthesized from the cytomegalovirus immediate early promoter (*CMV-IE*), the first exon and first intron (containing enhancer activity) of the chicken *β-actin* gene, and the 3′ splice acceptor site of the rabbit *β-globin* gene in place of the acceptor site of the chicken *β-actin* gene (Jun-ichi *et al.*, 1989; Hitoshi *et al.*, 1991). The *CAG* promoter was shown to be functional in BME26 (*R. microplus*) (Esteves *et al.*, 2008) and ISE6 cells (*I. scapularis*) by DsRed fluorescence (Kurtti *et al.*, 2008; Naranjo *et al.*, 2013). The *CMV-IE* promoter itself was capable of driving expression of transposase (SB10) from a plasmid containing the *Sleeping Beauty* vector, a *Tc1/mariner* group transposable element based on teleost fish (Ivics *et al.*, 1997), to integrate DsRed into ISE6 genomic DNA (Kurtti *et al.*, 2008). Also, the Simian Virus 40 (*SV40*) promoter was used to confer neomycin resistance in transfected ISE6 cells (Kurtti *et al.*, 2008). Constructs containing *CAG*, *CMV-IE*, or *SV40* promoters are widely available from Addgene and through commercial vendors, offering simplified access to tick researchers.

Cauliflower mosaic virus promoter

The cauliflower mosaic virus (CaMV) 35S promoter (343 bp), along with the 19S promoter, drives transcription of the entire CaMV viral genome (Bak and Emerson, 2020; Amack and Antunes, 2020). Machado-Ferreira *et al.* (2015) used *A. tumefasciens*-mediated gene transfer in BME/CTVM2

(*R. microplus*) cells to express EGFP–EGFP fusion or Salp15 (from *I. scapularis*)–EGFP fusion proteins under the CaMV 35S promoter. This promoter can also be found in the Addgene collection and is available from commercial vendors.

Untested potential promoter: 3xP3

A conserved genetic pathway that governs complex eye development in all metazoans is under the control of the Pax6 tissue-specific transcription factor (Callaerts *et al.*, 1997) and is considered to be a master switch (Gehring, 2002). This conservation among species led to the development of the artificial *3xP3* promoter (Berghammer *et al.*, 1999). The *3xP3* promoter consists of three Pax6 homodimer binding sites fused with a minimal *hsp70* promoter (about 120 bp) from *D. melanogaster* and can drive EGFP expression in the eyes of insects (Berghammer *et al.*, 1999; Horn *et al.*, 2000) (see Nolan and Hammond, Chapter 3, this volume).

The conserved role of Pax6 in metazoan eye development (Callaerts *et al.*, 1997) suggests that the *3xP3* promoter could be functional in most animals with eyes. Orthologues of *D. melanogaster Pax6* exist in the *I. scapularis* genome (ISCW003096), *Rhipicephalus sanguines* (XM_037656705.1) and *Dermacentor silvarum* (XM_037709500I). It is plausible that the *3xP3* would be functional in ticks that have eyes (such as *Ambylomma*, *Dermacentor* and *Rhipicephalus*). *3xP3* may be functional elsewhere in eyeless ticks of the genera *Ixodes* and *Haemaphysalis* spp. (such as in the synganglion) as *3xP3* drives expression in nervous tissue such as nerve cords in mosquitoes (Volohonsky *et al.*, 2017). However, there are exceptions where *3xP3* does not work, for instance in tephritid flies (Schetelig and Handler, 2013). This could be due to differences in eye development or that tephritid transcriptional machinery may not recognize the *D. melanogaster hsp70* minimal promoter. This was the case in the crustacean *Parhyale hawaiensis*, where the *Drosophila hsp70* promoter showed poor activity (Pavlopoulos and Averof, 2005). Therefore, *3xP3* with the *D. melanogaster hsp70* core promoter may not be functional in ticks and so replacing the *D. melanogaster* core promoter with a tick core promoter might be necessary.

19.3.5 Future identification of tick promoters

As tick transgenic technology develops, there will be a need to identify and validate tissue-, life-stage- and sex-specific promoters and enhancers for use in genetic studies and strategies for pathogen and vector control. Developmental transcriptomics studies, such as those performed in *Aedes aegypti* (Akbari *et al.*, 2013), have been useful in identifying sex-specific gene transcription, besides identifying key developmental genes. As it is not possible currently to identify sex in ticks prior to the adult life stage, one possible approach is to integrate a fluorescent reporter to a sex chromosome (for tick species with sex chromosomes) to label eggs, larvae and nymphs according to sex. Also, tissue-specific transcriptomics may reveal highly expressed genes within tissue types and their promoters (Matthews *et al.*, 2016). Similarly, transcriptomics to specific stimuli, such as blood intake, may permit the identification of inducible promoters.

Promoter, enhancer and silencer elements are often found in accessible chromatin DNA, which makes identifying these regions a useful first step in identifying putative promoters and enhancers (Shashikant and Ettensohn, 2019). An attractive approach that may work with limiting amounts of tissue is ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing). This technique, first described in 2013 (Buenrostro *et al.*, 2013), assesses DNA accessibility using a hyperactive Tn5 transposase that simultaneously cleaves accessible chromatin DNA, leaving a staggered cut, and then ligates high-throughput sequencing adapters to the cut (Shashikant and Ettensohn, 2019; Yan *et al.*, 2020). One advantage of using ATAC-seq over alternative techniques for chromatin accessibility such as DNase I hypersensitive sites sequencing (DNase-seq) and Formaldehyde-Assisted Isolation of

Regulatory Elements sequencing (FAIRE-seq) is that ATAC-seq requires a much smaller sample (500–50,000 cells) (Buenrostro *et al*., 2013; Yan *et al*., 2020). However, a limitation of ATAC-seq is that there are currently few bioinformatics tools custom-designed for ATAC-seq data (Yan *et al*., 2020). Still, the ability to use a much smaller sample size would also allow for the identification of tissue-specific promoter or enhancer sequences from dissected tick tissue.

19.4 Tick Transgenesis Strategies

CRISPR/Cas9-mediated gene editing functions by creating a double-stranded break in DNA, which triggers the DNA repair mechanisms in the cell, resulting in deletion or insertion at the cut site (Jinek *et al*., 2012) (see Concha and Papa, Chapter 7, this volume). The technique itself is straightforward and can be used in any species; however, the bottleneck to achieving a transformed organism usually occurs at the delivery stage. Cas9 and synthetic guide RNA must be delivered to the embryo (organismal transformation) or cells (for cell line transformation) for stable, heritable edits. Many non-model arthropods do not have an established embryo injection protocol to target the organism at its earliest life stage: a single cell. Here, we discuss potential strategies for delivery of the transformation reagents to embryos either by manipulating the embryo itself (injection, electroporation) or by delivering the reagents to embryos via gravid female (receptor-mediated endocytosis, bacterial and viral vectors) (Fig. 19.2).

19.4.1 Delivery of transgenic constructs

Embryo injections

Embryo injection remains the preferred approach for transgenesis, due to its high efficacy and increased likelihood of inducing heritable genetic alterations. Injecting directly into freshly deposited eggs prior to differentiation enables the embryonic germline to be modified. Originally developed in *D. melanogaster*, the embryo injection protocol has been successfully modified and applied to a variety of insect and non-insect arthropods generating heritable changes in the genome. However, in non-model arthropods, such as ticks, the absence of a well-established protocol for embryo injection has stymied genome editing (Nuss *et al*., 2021).

As mentioned above (Section 9.2), a wax layer protects tick eggs from desiccation and also makes embryo injection almost impossible. Additionally, microinjections are complicated by the high intra-oval pressure, as embryos burst upon needle penetration without appropriate egg conditioning. Therefore, the wax layer must first be removed (or prevented from being deposited) to ensure successful embryo injections to avoid needle breakage and to allow for controlled desiccation, which is necessary to alleviate intra-oval pressure. We recently developed an embryo injection protocol for *I. scapularis* by first dissecting or emptying the wax gland (Gené's organ) from the egg-laying female to obtain unwaxed or slightly waxed eggs (Sharma *et al*., 2020). The intra-oval pressure was then decreased by treating eggs with sodium chloride. This protocol was then utilized to inject CRISPR components (sgRNA and Cas9) targeting multiple genes in embryos within 12–18 h AEL. Our results confirmed the feasibility of gene editing in ticks using embryo injections (Sharma *et al*., 2020).

Gravid adult injections

RECEPTOR-MEDIATED OVARY TRANSDUCTION OF CARGO. Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control) is an alternative strategy to deliver the CRISPR construct into developing oocytes by injecting gravid females (Chaverra-Rodriguez *et al*., 2018) (see Terradas *et al*., Chapter 6, this volume). CRISPR/Cas9 cargo is delivered into the embryos using peptide ligands derived from the *D. melanogaster* yolk proteins precursor (YPPs) or similar yolk proteins that are fused to the Cas9 protein. Along with the Cas9–sgRNA cargo, an endosomal escape reagent
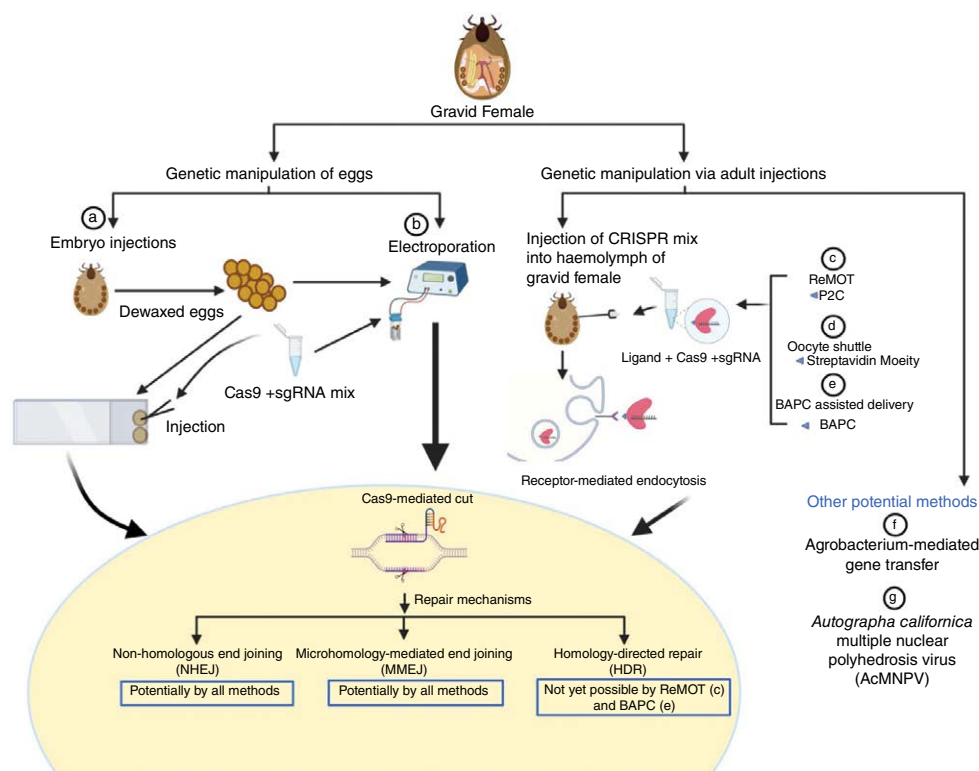
Fig. 19.2. Potential strategies for tick transformation. The most preferred approach for germline transformation has been embryo injection; however, electroporation has also been successfully used for RNAi and may also work for gene knockout. Other approaches that utilize receptor-mediated endocytosis (c, d, e) could be used by injecting gravid females (see Terradas *et al.*, Chapter 6, this volume). Bacterial and viral vectors may also potentially be used for the delivery of CRISPR/Cas9 constructs. Created by Biorender.com

(chloroquine or saponin) is used to aid in the release of the YPP complex from endosomes into the oocyte cytoplasm (Chaverra-Rodriguez *et al.*, 2018). Once injected into the gravid female haemolymph, the cargo is taken up by oocytes by receptor-mediated endocytosis using the YPP ligand. Editing occurs in these oocytes, and then females lay eggs containing the targeted gene mutation. Although this method was initially tested in the mosquito, *Aedes aegypti*, it has since been applied to a variety of insect species (Chaverra-Rodriguez *et al.*, 2018; Dermauw *et al.*, 2020; Heu *et al.*, 2020 Macias *et al.*, 2020; Shirai and Daimon, 2020), demonstrating its broad applicability in insects.

We successfully utilized ReMOT Control technology to create mutations in *I. scapularis*

using Cas9-YPP, sgRNAs and saponin (Sharma *et al.*, 2020). However, this technology is limited to gene knockout as there is currently no mechanism for introducing guide templates for homology-directed repair (HDR) for gene knock-ins (Chaverra-Rodriguez *et al.*, 2018; Macias *et al.*, 2020).

OOCYTE SHUTTLE. Oocyte shuttle, a similar approach to ReMOT Control, was employed to genetically transform *Xenopus laevis* (Runngger *et al.*, 2017). This technique uses oocyte shuttle proteins containing a *Xenopus* vitellogenin protein fragment attached to a streptavidin moiety to bind biotinylated DNA. When injected into the bloodstream of adult females, the protein and DNA complex are taken up by the ovary via

receptor-mediated endocytosis. This approach, like ReMOT Control, should function in all vitellogenic species, since the vitellogenin ligand is present in the protein and it binds to receptors on the oocyte's surface. An oocyte shuttle protocol might work in ticks, allowing researchers to conduct gene knock-in experiments, provided a repair template can be bound to the vitellogenin ligand.

BRANCHED AMPHIPHILIC PEPTIDE CAPSULES (BAPC)-ASSISTED TARGETED DELIVERY. BAPC is a peptide-based nano-assembly that has shown potential as a delivery vehicle for dsRNA and plasmid in *Tribolium castaneum* and *Acyrthosiphon pisum* (pea aphid). BAPC facilitates the gradual release of dsRNA into the cell and protects it from nucleases, increasing RNAi efficiency (Sukthankar *et al.*, 2014; Avila *et al.*, 2018; Hunter *et al.*, 2018).

Hunter *et al.* (2018) described the use of BAPC for delivery of Cas9 protein and sgRNA for gene editing in *Diaphorina citri* (Asian citrus psyllid). Fifth-instar nymphs and adults were injected with the BAPC-assisted delivery of CRISPR construct to knock out the *thioredoxin* gene (TRX). The authors suggested that the knockout was heritable. This technique has not yet been tested in other insects and its efficacy in other arthropod species has not been established. In principle, this method may potentially be explored for functional studies in ticks for both somatic editing and germline transformation.

19.4.2 Other potential methods for tick transgenics

Electroporation of plasmids encoding Cas9 or Cas9 proteins

Electroporation is a powerful transfection technique that is widely used for gene expression studies. It temporarily increases the permeability of the cell membrane, allowing proteins and nucleic acids to enter the cell. Even though its efficacy is limited and significant cell death occurs, it has been used for gene editing in both vertebrates and invertebrates (Thomas, 2003; Zawadzki *et al.*, 2012; Ando and Fujiwara, 2013; Liu *et al.*, 2017; Jamison *et al.*, 2018).

In ticks, electroporation has been used for the delivery of dsRNA into eggs and mature stages for gene knockdown. In *I. scapularis*, *Phospholipase A2* (*PLA2*), cytoplasmic *cystatin*, *Syntaxin-5* (*STX5*), *β-actin* and *calreticulin* genes were targeted for knockdown in eggs and unfed nymphs by delivering dsRNA via electroporation. All of these genes were significantly silenced, demonstrating the utility of electroporation as a high-throughput nucleic acid delivery technique (Karim *et al.*, 2010).

Silencing of *Protein Kinase B* (AKT) and *glycogen kinase synthase* (*GSK*) genes in dewaxed *R. microplus* eggs has been demonstrated using electroporation (Ruiz *et al.*, 2015). Approximately 50% reduction in gene activity was reported. However, the use of heptane and hypochlorite to remove wax from the eggs had a detrimental effect on egg survival. In unaltered eggs, dsRNA was not absorbed as wax coating impeded the electrical pulse conductance onto the eggshell (Ruiz *et al.*, 2015).

We employed electroporation to deliver a CMV–CAGGS plasmid construct driving DsRed in *I. scapularis* eggs and observed fluorescence in hatched larvae (unpublished data). The CAGGS plasmid has also been successfully used in ISE6 cell lines. To obtain dewaxed eggs for this experiment, the Gené's organ was ablated from egg-laying females (as described in section 19.4.1 under 'Embryo injections'), which avoids chemical wax removal steps that incur increased embryo mortality. Our results, along with other studies in *I. scapularis* and *R. microplus*, demonstrate the feasibility of delivering plasmids into tick eggs via electroporation. However, our preliminary work with electroporation of sgRNA and Cas9 for gene editing did not show any Cas9-mediated cuts, and all sequences were wild-type (unpublished data), suggesting that there are further challenges to developing electroporation as a viable transformation technique in ticks.

Agrobacterium tumefaciens-mediated gene transfer

Agrobacterium tumefaciens is a naturally occurring soil phytopathogen that causes crown gall disease in plants. The extraordinary ability of *Agrobacterium* to transfer its genetic material to a host cell has enabled it to evolve from a phytopathogen to a potent transgenic vector (reviewed by Nester, 2015). Transformation is facilitated in plants with a single-stranded DNA (ssDNA), T-DNA, that induces the plant to form a tumour (gall) (Lacroix and Citovsky, 2019; Thompson *et al.*, 2020). This ssDNA version of T-DNA is exported from the bacterium to the host cell, eventually being integrated into the host chromosomal DNA (Lacroix and Citovsky, 2019).

In ticks, suspensions of *A. tumefaciens* have been used to transfer EGFP plasmids fused with *EGFP* or *Salp15* gene into *I. scapularis* and *R. microplus* (Baldridge *et al.*, 2007; Machado-Ferreira *et al.*, 2015). Although mortality was high (approximately 30% of larvae survived post-treatment) and no EGFP fluorescence was detected in the extracted intestinal tissue from the surviving larvae, transgene expression was detected by nested RT-PCR targeting *EGFP* or the *Salp15* gene. No contaminating *A. tumefaciens* DNA was detected after two rounds of nested PCR targeting either the *16s rRNA* or *virD* genes, indicating the efficiency of transgene expression (Machado-Ferreira *et al.*, 2015).

Autographa californica multiple nuclear polyhedrosis virus (AcMNPV)

AcMNPV is the type species of the *Alphabaculovirus* genus, with a double-stranded circular DNA genome of 134 kb and 150 kb open reading frames. The ability of AcMNPV to infect insect cells has resulted in its use in a variety of protein expression systems (Kitajima and Takaku, 2008). As noted above in the section on the *polyhedrin* promoter, You *et al.* (2003) topically applied recombinant AcMNPV to the dorsal sides of *H. longicornis* larvae and adults to express a V5-His-tagged *chitinase* under the *polyhedrin* promoter.

Recombinant V5 was detected by western blotting and immunofluorescence staining. Despite this success, AcMNPV infection resulted in the eventual death of infected *H. longicornis* (You *et al.*, 2003). The development of a replication-deficient baculovirus in a system similar to the current second and third generation lentiviral expression systems would be useful as a non-lethal transformation method. This could involve separating the AcMNPV genome into envelope and packaging plasmids, with the transgene of interest being placed in a transfer vector (Addgene, 2021). To address the potential toxicity of the hyper-transcriptional activity of the polyhedrin promoter, transgene expression under a weaker promoter may decrease mortality.

19.5   Conclusions

In this chapter, we have discussed the current knowledge, available tools and future directions in tick transgenics. While many insect systems have successfully utilized genetic transformation to explore gene function and to develop potential vector and pathogen control measures, the lack of transformation protocols for ticks has restricted research on tick biology and the tick–host–pathogen interface (de La Fuente, 2021). The development of an injection protocol for introducing transgenic constructs directly to embryos will accelerate tick research. Being able to manipulate tick embryos will potentially open new research directions such as site-specific genome targeting by recombinant mediated cassette exchange using CRISPR, transgenic strains for population control such as the use of dominant lethal allele system (similar to RIDL®) (Alphey *et al.*, 2013), and stable endosymbiont expression to study tick–symbiont interactions, to name just a few. Increased understanding of embryology will allow further refinement of the embryo injection protocol which will increase embryo survival and transformation efficiency. Potentially, the use of techniques such as microCT that allow real-time visualization of internal

morphology of developing embryos would help localize the germ cells and might provide landmarks for anterior/posterior axis formation. Additional methods for introducing transgenic constructs in gravid female haemolymph that could be taken up by the developing embryos within the ovaries (outlined in this chapter) are also of importance because of the ease of injection. The DNA delivery methods outlined in this chapter will further benefit from endogenous and non-endogenous promoters validated from prior work in tick cell lines or newly identified and validated as markers of successful transformation, transgenic line maintenance and gene function studies, as well as developing efforts for population replacement (through pathogen refractory genes), or vector control. In addition to the promoters identified in tick cells, tissue-, sex- and life-stage-specific promoters will need to be identified and validated for gene function studies as well as vector and pathogen control strategies. We have highlighted some potential methods for identifying promoter and enhancer elements such as transcriptomics and ATAC-seq. With tick transformation protocols being developed, the development of functional promoter constructs for ticks is the proximal step towards accelerating tick research and vector control methods.

References

Addgene (2021) *Lentiviral Guide*. Available at https://www.addgene.org/guides/lentivirus/ (accessed 26 May 2021)

Aeschlimann, A. (1958) Développement embryonnaire d'*Ornithodorus moubata* (Murray) et transmission transovarienne de *Borrelia duttoni*. [Translation]. *Acta Tropica* 15, 15–64.

Akbari, O.S., Antoshechkin, I., Amrhein, H., Williams, B., Sandler, J. *et al.* (2013) The developmental transcriptome of the mosquito *Aedes aegypti*, an invasive species and major arbovirus vector. *G3: Gene, Genome, Genetics* 3, 1493–1509. doi: 10.1534/g3.113.006742

Akiyama-Oda, Y. and Oda, H. (2003) Early patterning of the spider embryo: a cluster of mesenchymal cells at the cumulus produces Dpp signals received by germ disc epithelial cells. *Development* 30(9), 1735–1747. doi: 10.1242/dev.00390

Almazán, C., Kocan, K.M., Bergman, D.K., Garcia-Garcia, J.C., Blouin, E.F. *et al.* (2003) Identification of protective antigens for the control of *Ixodes scapularis* infestations using cDNA expression library immunization. *Vaccine* 21 (13-14), 1492–1501. doi: 10.1016/S0264-410X(02)00683-7

Alphey, L., McKemey, A., Nimmo, D., Neira Oviedo, M., Lacroix, R. *et al.* (2013) Genetic control of *Aedes mosquitoes*. *Pathogens and Global Health* 107(4), 170–179. doi: 10.1179/2047773213Y.0000000095

Amack, S.C. and Antunes, M.S. (2020) CaMV35S promoter – a plant biology and biotechnology workhorse in the era of synthetic biology. *Current Plant Biology* 24, 100–179. doi :10.1016/j.cpb.2020.100179

Ando, T. and Fujiwara, H. (2013) Electroporation-mediated somatic transgenesis for rapid functional analysis in insects. *Development* 140, 454–458. doi: 10.1242/dev.085241

Arosio, P., Ingrassia, R. and Cavadini, P. (2009) Ferritins: a family of molecules for iron storage, antioxidation and more. *Biochimica et biophysica acta* 1790(7), 589–599. doi: 10.1016/j.bbagen.2008.09.004

Arthur, D.R. (1953) The morphology of the British prostriata with particular reference to *Ixodes hexagonus* leach. *II. Parasitology* 42, 161–186. doi: 10.1017/S0031182000084432

Artigas-Jerónimo, S., Villar, M., Cabezas-Cruz, A., Valdés, J.J., Estrada-Peña, A. *et al.* (2018) Functional evolution of subolesin/akirin. *Frontiers in Physiology* 9, 1612. doi: 10.3389/fphys.2018.01612.

Avila, L.A., Chandrasekar, R., Wilkinson, K.E., Balthazor, J., Heerman, M. *et al.* (2018) Delivery of lethal dsRNAs in insect diets by branched amphiphilic peptide capsules. *Journal of Controlled Release* 273, 139–146. doi: 10.1016/j.jconrel.2018.01.010

Bak, A. and Emerson, J.B. (2020) Cauliflower mosaic virus (CaMV) biology, management, and relevance to GM plant detection for sustainable organic agriculture. *Frontiers in Sustainable Food Systems* 4, 21. doi: 10.3389/fsufs.2020.00021

Baldridge, G.D., Kurtti, T.J., Burkhardt, N., Baldridge, A.S., Nelson, C.M. *et al.* (2007) Infection of *Ixodes scapularis* ticks with *Rickettsia monacensis* expressing green fluorescent protein: a model system. *Journal of Invertebrate Pathology* 94(3), 163–174. doi: 10.1016/j.jip.2006.10.003

Berghammer, A.J., Klingler, M. and Wimmer, E.A. (1999) A universal marker for transgenic insects. *Nature* 402, 370–371. doi: 10.1038/46463

Booth, T.F. (1989) Wax lipid secretion and ultrastructural development in the egg-waxing (Gené's) organ in ixodid ticks. *Tissue and Cell* 21, 113–122. doi: 10.1016/0040-8166(89)90026-8

Brilliant, M.H. (2001) The mouse p (pink-eyed dilution) and human P genes, oculocutaneous albinism type 2 (OCA2), and melanosomal pH. *Pigment Cell Research* 14, 86–93. doi: 10.1034/j.1600-0749.2001.140203.x

Brinton, L.P. and Oliver, J.H. (1971) Fine structure of oogonial and oocyte development in *Dermacentor andersoni* Stiles (Acari: Ixodidae). *Journal of Parasitology* 57(4), 720–747.

Buenrostro, J.D., Giresi, P.G., Zaba, L.C., Chang, H.Y. and Greenleaf, W.J. (2013) Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nature Methods* 10, 1213–1218. doi: 10.1038/nmeth.2688

Callaerts, P., Halder, G. and Gehring, W.J. (1997) PAX-6 in development and evolution. *Annual Review of Neuroscience* 20, 483–532. doi: 10.1146/annurev.neuro.20.1.483

Campbell, A. (1979) Ecology of the American dog tick, *Dermacentor variabilis* in southwestern Nova Scotia. *Recent Advances in Acarology* 2, 135–143.

Campos, E., Moraes, J., Façanha, A.R., Moreira, É., Valle, D. *et al.* (2006) Kinetics of energy source utilization in *Boophilus microplus* (Canestrini, 1887) (Acari: Ixodidae) embryonic development. *Veterinary Parasitology* 138(3–4), 349–357. doi: 10.1016/j.vetpar.2006.02.004

Centers for Disease Control and Prevention (2015) How many people get Lyme disease? Available at https://www.cdc.gov/lyme/stats/humancases.html (accessed 15 April 2022)

Chaverra-Rodriguez, D., Macias, V.M., Hughes, G.L., Pujhari, S., Suzuki, Y. *et al.* (2018) Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing. *Nature Communications* 9(1), 3008. doi: 10.1038/s41467-018-05425-9

Chetverikov, P.E. and Desnitskiy, A.G. (2016) A study of embryonic development in eriophyoid mites (Acariformes, Eriophyoidea) with the use of the fluorochrome DAPI and confocal microscopy. *Experimental and Applied Acarology* 68, 97–111. doi: 10.1007/s10493-015-9982-4

de la Fuente, J. (2021) Translational biotechnology for the control of ticks and tick-borne diseases. *Ticks and Tick Borne Diseases* 12(5), 101738. doi: 10.1016/j.ttbdis.2021.101738

de Oliveira, P.R., Mathias, M.I.C. and Bechara, G.H. (2007) Vitellogenesis in the tick *Amblyomma triste* (Koch, 1844) (Acari: Ixodidae). *Role for pedicel cells. Veterinary Parasitology* 143, 134–139. doi: 10.1016/j.vetpar.2006.08.013

Dermauw, W., Jonckheere, W., Riga, M., Livadaras, I., Vontas, J. *et al.* (2020) Targeted mutagenesis using CRISPR-Cas9 in the chelicerate herbivore *Tetranychus urticae*. *Insect Biochemistry and Molecular Biology* 120, 103347. doi: 10.1016/j.ibmb.2020.103347

Eggleston, P. and Meredith, J.M. (2014) Docking systems for site-directed transgene integration. In: Benedict, M.Q. (ed.) *Transgenic Insects: Techniques and Applications*. CABI International, Wallingford, UK, pp. 51–65. doi:10.1079/9781780644516.0000

El-Kammah, K.M., Adham, F.K., Tadross, N.R. and Osman, M. (1982) Embryonic development of the camel tick *Hyalomma dromedarii* (Ixodoidea: Ixodidae). *International Journal of Acarology* 8, 47–54.

Esteves, E., Lara, F.A., Lorenzini, D.M., Costa, G.H.N., Fukuzawa, A.H. *et al.* (2008) Cellular and molecular characterization of an embryonic cell line (BME26) from the tick *Rhipicephalus* (*Boophilus*) *microplus*. *Insect Biochemistry and Molecular Biology* 38(5), 568–580. doi: 10.1016/j.ibmb.2008.01.006

Fagotto, F., Hess, E. and Aeschlimann, A. (1988) The early embryonic development of the argasid tick *Ornithodoros moubata* (Acarina: Ixodoidea: Argasidae). *Entomologia Generalis* 13, 1–8

Foe, V.E. and Alberts, B.M. (1983) Studies of nuclear and cytoplasmic behavior during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. *Journal of Cell Science* 61, 31–70. doi: 10.1242/jcs.61.1.31

Friesen, K.J., Dixon, M. and Lysyk, T.J. (2016) Embryo development and morphology of the Rocky Mountain wood tick (Acari: Ixodidae). *Journal of Medical Entomology* 53, 279–289. doi: 10.1093/jme/tjv193

Galay, R.L., Aung, K.M., Umemiya-Shirafuji, R., Maeda, H., Matsuo, T. *et al.* (2013) Multiple ferritins are vital to successful blood feeding and reproduction of the hard tick *Haemaphysalis longicornis*. *Journal of Experimental Biology* 216, 1905–1915. doi: 10.1242/jeb.081240

Galindo, R.C., Doncel-Pérez, E., Zivkovic, Z., Naranjo, V., Gortazar, C. *et al.* (2009) Tick subolesin is an ortholog of the akirins described in insects and vertebrates. *Developmental and Comparative Immunology* 33(4), 612–617. doi: 10.1016/j.dci.2008.11.002

Gehring, W.J. (2002) The genetic control of eye development and its implications for the evolution of the various eye-types. *International Journal of Developmental Biology* 46(1), 65–73. doi: pubmed.ncbi. nlm.nih.gov/11902689/

Gilbert, S.F. (2000) An introduction to early developmental processes. In: *Developmental Biology*. Sinaeur, Sunderland, Massachusetts, pp. 195–197.

Guerrero, F.D. and Nene, V.M. (2008) Gene structure and expression of a pyrethroid-metabolizing esterase, CzEst9, from a pyrethroid resistant Mexican population of *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae). *Journal of Medical Entomology* 45(4), 677–685. doi: 10.1093/jmedent/45.4.677

Gulia-Nuss, M., Nuss, A.B., Meyer, J.M., Sonenshine, D.E., Roe, R.M. *et al.* (2016) Genomic insights into the *Ixodes scapularis* tick vector of Lyme disease. *Nature Communications* 7 (1), 10507. doi: 10.1038/ ncomms10507

Hernandez, E.P., Kusakisako, K., Hatta, T. and Tanaka, T. (2019) Characterization of an iron-inducible *Haemaphysalis longicornis* tick-derived promoter in an *Ixodes scapularis*-derived tick cell line (ISE6). *Parasites & Vectors* 12(1), 321. doi: 10.1186/s13071-019-3574-9

Heu, C.C., Mccullough, F.M., Luan, J. and Rasgon, J.L. (2020) CRISPR-Cas9-based genome editing in the silverleaf whitefly (*Bemisia tabaci*). *CRISPR Journal* 3(2), 89–96. doi: 10.1089/crispr.2019.0067

Hitoshi, N., Ken-ichi, Y. and Jun-ichi, M. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108(2), 193–199. doi: 10.1016/0378-1119(91)90434-D

Horn, C., Jaunich, B. and Wimmer, E.A. (2000) Highly sensitive, fluorescent transformation marker for *Drosophila* transgenesis. *Development Genes and Evolution* 210(12), 623–629. doi: 10.1007/ s004270000111

Hunter, W., Gonzalez, M. and Tomich, J. (2018) BAPC-assisted CRISPR/Cas9 system: targeted delivery into adult ovaries for heritable germline gene editing (Arthropoda: Hemiptera). *bioRxiv* 478743. doi: 10.1101/478743

Ivics, Z., Hackett, P.B., Plasterk, R.H. and Izsvák, Z. (1997) Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. *Cell* 91(4), 501–510. doi: 10.1016/ S0092-8674(00)80436-5

Jamison, B.V., Thairu, M.W. and Hansen, A.K. (2018) Efficacy of in vivo electroporation on the delivery of molecular agents into aphid (Hemiptera: Aphididae) ovarioles. *Journal of Insect Science* 18, 49. doi:10.1093/jisesa/iey041

Jia, N., Wang, J., Shi, W., Du, L., Sun, Y. *et al.* (2020) Large-scale comparative analyses of tick genomes elucidate their genetic diversity and vector capacities. *Cell* 182(5), 1328–1340.e13. doi: 10.1016/j. cell.2020.07.023

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337(6096), 816–821. doi: 10.1126/science.1225829

Jongejan, F. and Uilenberg, G. (2004) The global importance of ticks. *Parasitology* 129, S3–S14. doi:10.1017/ S0031182004005967

Jun-ichi, M., Satoshi, T., Kimi, A., Fumi, T., Akira, T. *et al.* (1989) Expression vector system based on the chicken β-actin promoter directs efficient production of interleukin-5. *Gene* 79(2), 269–277. doi: 10.1016/0378-1119(89)90209-6

Kakuda, H., Koga, T., Mori, T. and Shiraishi, S. (1994) Ultrastructure of the tubular accessory gland in *Haemaphysalis longicornis* (Acari: Ixodidae). *Journal of Morphology* 221, 65–74. doi: 10.1002/ jmor.1052210105

Karim, S., Troiano, E. and Mather, T.N. (2010) Functional genomics tool: gene silencing in *Ixodes scapularis* eggs and nymphs by electroporated dsRNA. *BMC Biotechnology* 10, 1. doi: 10.1186/1472-6750-10-1

Kitajima, M. and Takaku, H. (2008) Induction of antitumor acquired immunity by baculovirus *Autographa californica* multiple nuclear polyhedrosis virus infection in mice. *Clinical and Vaccine Immunology* 15, 376–378. doi: 10.1128/CVI.00285-07

Kocan, K.M., Blouin, E. and de la Fuente, J. (2011) RNA interference in ticks. *Journal of Visualized Experiments* 47, 2474. doi: 10.3791/2474

Kurtti, T.J., Mattila, J.T., Herron, M.J., Felsheim, R.F., Baldridge, G.D. *et al.* (2008) Transgene expression and silencing in a tick cell line: a model system for functional tick genomics. *Insect Biochemistry and Molecular Biology* 38(10), 963–968. doi: 10.1016/j.ibmb.2008.07.008

Kusakisako, K., Ido, A., Masatani, T., Morokuma, H., Hernandez, E.P. *et al.* (2018) Transcriptional activities of two newly identified *Haemaphysalis longicornis* tick-derived promoter regions in the Ixodes scapularis tick cell line (ISE6). *Insect Molecular Biology* 27(5), 590–602. doi: 10.1111/imb.12497

Lacroix, B. and Citovsky, V. (2019) Pathways of DNA transfer to plants from *Agrobacterium tumefaciens* and related bacterial species. *Annual Review of Phytopathology* 57, 231–251. doi: 10.1146/annurev-phyto-082718-100101

Larson, S.R. and Paskewitz, S.M. (2016) Teratological nymphal *Ixodes scapularis* (Acari: Ixodidae) from Wisconsin. *Journal of Medical Entomology* 53(2), 477–479. doi: 10.1093/jme/tjv198

Laumann, M., Bergmann, P., Norton, R.A. and Heethoff, M. (2010) First cleavages, preblastula and blastula in the parthenogenetic mite *Archegozetes longisetosus* (Acari, Oribatida) indicate holoblastic rather than superficial cleavage. *Arthropod Structure and Development* 39, 276–286. doi: 10.1016/j.asd.2010.02.003

Li, M., Bui, M., Yang, T., Bowman, C.S., White, B.J. and Akbari, O.S. (2017) Germline Cas9 expression yields highly efficient genome engineering in a major worldwide disease vector, *Aedes aegypti*. *Proceedings of the National Academy of Sciences USA* 114, E10540–E10549. doi: 10.1073/pnas.1711538114

Liu, C., Zhang, Li., Liu, H. and Cheng, K. (2017) Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications. *Journal of Controlled Release* 266, 17-26. doi: 10.1016/j.jconrel.2017.09.012

Liu, L., Cheng, T. and Yan, F. (2016) Expression pattern of subA in different tissues and blood-feeding status in *Haemaphysalis flava*. *Experimental and Applied Acarology* 70(4), 511–522. doi: 10.1007/s10493-016-0088-4

Lubinga, J.C., Tuppurainen, E.S., Mahlare, R., Coetzer, J.A., Stoltsz, W.H. and Venter, E.H. (2015) Evidence of transstadial and mechanical transmission of lumpy skin disease virus by *Amblyomma hebraeum* ticks. *Transboundary and Emerging Diseases* 62(2), 174-82. doi: 10.1111/tbed.12102

Machado-Ferreira, E., Balsemão-Pires, E., Dietrich, G., Hojgaard, A., Vizzoni, V.F. *et al.* (2015) Transgene expression in tick cells using *Agrobacterium tumefaciens*. *Experimental and Applied Acarology* 67(2), 269–287. doi: 10.1007/s10493-015-9949-5

Macias, V.M., McKeand, S., Chaverra-Rodriguez, D., Hughes, G.L., Fazekas, A. *et al.* (2020) Cas9-mediated gene-editing in the malaria mosquito *Anopheles stephensi* by ReMOT Control. *G3: Genes, Genomes, Genetics* 10(4), 1353–1360. doi: 10.1534/G3.120.401133

Matheson R. (1950) *Medical Entomology*, 2nd edn. Comstock Publishing Company, Ithaca, New York.

Matthews, B.J. and Vosshall, L.B. (2020) How to turn an organism into a model organism in 10 'easy' steps. *Journal of Experimental Biology* 223 (Suppl. 1), jeb.218198. doi: 10.1242/jeb.218198

Matthews, B.J., McBride, C.S., DeGennaro, M., Despo, O. and Vosshall, L.B. (2016) The neurotranscriptome of the *Aedes aegypti* mosquito. *BMC Genomics* 17(1), 32. doi: 10.1186/s12864-015-2239-0

McGregor, A.P., Hilbrant, M., Pechmann, M., Schwager, E.E., Prpic, N.M. *et al.* (2008) *Cupiennius salei* and *Achaearanea tepidariorum*: spider models for investigating evolution and development. *BioEssays* 30, 487–498. doi: 10.1002/bies.20744

Mehlhorn, H., Jabbar, A. and Petney, T. (2016) Ticks. In: Mehlhorn, H. (ed.) *Encyclopedia of Parasitology*. Springer, Berlin, Heidelberg. doi: 10.1007/978-3-662-43978-4_3181

Mittmann, B. and Wolff, C. (2012) Embryonic development and staging of the cobweb spider *Parasteatoda tepidariorum* C. L. Koch, 1841 (syn.: *Achaearanea tepidariorum*; Araneomorphae; Theridiidae). *Development Genes and Evolution* 222, 189–216. doi: 10.1007/s00427-012-0401-0

Moreira, L.A., Edwards, M.J., Adhami, F., Jasinskiene, N., James, A.A., *et al.* (2000) Robust gut-specific gene expression in transgenic *Aedes aegypti* mosquitoes. *Proceedings of the National Academy of Sciences USA* 97(20), 10895–10898. doi: 10.1073/pnas.97.20.10895

Moreira, L.A., Ito, J., Ghosh, A., Devenport, M., Zieler, H. *et al.* (2002) Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes. *Journal of Biological Chemistry* 277(43), 40839–40843. doi: 10.1074/jbc.M206647200

Naranjo, V., Ayllón, N., La Pérez de Lastra, J.M., Galindo, R.C., Kocan, K.M. *et al.* (2013) Reciprocal regulation of NF-kB (Relish) and Subolesin in the tick vector, *Ixodes scapularis*. *PLoS ONE* 8(6), e65915. doi: 10.1371/journal.pone.0065915

Nelder, M.P., Russell, C.B., Sheehan, N.J., Sander, B., Moore, S. *et al.* (2016) Human pathogens associated with the blacklegged tick *Ixodes scapularis*: a systematic review. *Parasites and Vectors* 9, 265. doi: 10.1186/s13071-016-1529-y

Nester, E.W. (2015) *Agrobacterium*: nature's genetic engineer. *Frontiers in Plant Science* 5, 730. doi: 10.3389/fpls.2014.00730

Nuss, A., Sharma, A. and Gulia-Nuss, M. (2021) Genetic manipulation of ticks: a paradigm shift in tick and tick-borne diseases research. *Frontiers in Cellular and Infection Microbiology* 11, 678037. doi: 10.3389/fcimb.2021.678037

Ogihara, M. and Taylor, D. (2014) Female reproductive system: anatomy, physiology, and molecular biology. In: Sonenshine, D.E. and Roe, R.M. (eds) *Biology of Ticks*, Vol. 1, 2nd edn. Oxford University Press, Madison, New York, pp. 449–483.

Pavlopoulos, A. and Averof, M. (2005) Establishing genetic transformation for comparative developmental studies in the crustacean *Parhyale hawaiensis*. *Proceedings of the National Academy of Sciences USA* 102(22), 7888–7893. doi: 10.1073/pnas.0501101102

Pressesky, M. (1952) *A study of the embryonic development of the Rocky Mountain spotted fever tick Dermacentor andersoni (Stiles)*. MS Thesis, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

Qin, J.Y., Zhang, L., Clift, K.L., Hulur, I., Xiang, A.P. *et al.* (2010) Systematic comparison of constitutive promoters and the doxycycline-inducible promoter. *PLoS ONE* 5(5), e10611. doi: 10.1371/journal.pone.0010611

Ramachandran, A., Bashyam, M.D., Viswanathan, P., Ghosh, S., Kumar, M.S. and Hasnain, S.E. (2001) The bountiful and baffling baculovirus: the story of polyhedrin transcription. *Current Science* 81, 998–1010.

Ruiz, N., De Abreu, L.A., Parizi, L.F., Kim, T.K., Mulenga, A. *et al.* (2015). Non-invasive delivery of dsRNA into de-waxed tick eggs by electroporation. *PLoS ONE* 10(6). doi: 10.1371/journal.pone.0130008

Rungger, D., Muster, L., Georgiev, O. and Rungger-Brändle, E. (2017) Oocyte shuttle, a recombinant protein transporting donor DNA into the *Xenopus* oocyte in situ. *Biology Open* 6, 290–295. doi: 10.1242/bio.022376

Santos, V.T., Ribeiro, L., Fraga, A., de Barros, C.M., Campos, E. *et al.* (2013) The embryogenesis of the tick *Rhipicephalus* (*Boophilus*) *microplus*: the establishment of a new chelicerate model system. *Genesis* 51(12), 803–818. doi: 10.1002/dvg.22717

Schetelig, M.F. and Handler, A.M. (2013) A functional comparison of the 3xP3 promoter by Recombinase-Mediated Cassette Exchange in *Drosophila* and a tephritid fly, *Anastrepha suspensa*. *G3: Genes, Genomes, Genetics* 3(4), 687–693. doi: 10.1534/g3.112.005488

Sharma, A., Pham, M.N., Reyes, J.B., Chana, R., Yim, W.C. *et al.* (2020) Cas9-mediated gene-editing in the black-legged tick, *Ixodes scapularis*, by embryo injection and ReMOT Control. *iScience* 25, 103781. doi: 10.2139/ssrn.3691041

Shashikant, T. and Ettensohn, C.A. (2019) Genome-wide analysis of chromatin accessibility using ATAC-seq. *Methods in Cell Biology* 151, 219–235. doi: 10.1016/bs.mcb.2018.11.002

Shirai, Y. and Daimon, T. (2020) Mutations in cardinal are responsible for the red-1 and peach eye color mutants of the red flour beetle *Tribolium castaneum*. *Biochemical and Biophysical Research Communications* 529, 372–378. doi: 10.1016/j.bbrc.2020.05.214

Soghigian, J., Ridge, G.E., Stafford, K.C. and Molaei, G. (2017) The first evidence of nanism in *Ixodes* (*Ixodes*) *scapularis* (Acari: Ixodidae), found parasitizing a human host. *Journal of Medical Entomology* 54(5), 1224–1228. doi: 10.1093/jme/tjx111

Sukthankar, P., Avila, L.A., Whitaker, S.K., Iwamoto, T., Morgenstern, A. *et al.* (2014) Branched amphiphilic peptide capsules: cellular uptake and retention of encapsulated solutes. *Biochimica et Biophysica Acta – Biomembranes* 1838(9), 2296–2305. doi: 10.1016/j.bbamem.2014.02.005

Thomas, J.L. (2003) Electroporation, an alternative to biolistics for transfection of *Bombyx mori* embryos and larval tissues. *Journal of Insect Science* 3, 1–12. doi: 10.1673/031.003.1701

Thompson, M.G., Moore, W.M., Hummel, N.F.C., Pearson, A.N., Barnum, C.R. *et al.* (2020) *Agrobacterium tumefaciens* : a bacterium primed for synthetic biology. *BioDesign Research* 2020, 1–16. doi: 10.34133/2020/8189219

Tuckow, A.P. and Temeyer, K.B. (2015) Discovery, adaptation and transcriptional activity of two tick promoters: construction of a dual luciferase reporter system for optimization of RNA interference in *Rhipicephalus* (*Boophilus*) *microplus* cell lines. *Insect Molecular Biology* 24(4), 454–466. doi: 10.1111/imb.12172

Volohonsky, G., Hopp, A., Saenger, M., Soichot, J., Scholze, H. *et al.* (2017) Transgenic expression of the anti-parasitic factor TEP1 in the malaria mosquito *Anopheles gambiae*. *PLoS Pathogens* 13(1), e1006113. doi: 10.1371/journal.ppat.1006113

Wagner, J. (1894) Die Embryonalentwicklung von Ixodes calcaratus. *Bir Trav Soc Nat St Petersb* 24, 214–246.

Wolff, C. and Hilbrant, M. (2011) The embryonic development of the central American wandering spider *Cupiennius salei*. *Frontiers in Zoology* 8, 15. doi: 10.1186/1742-9994-8-15

Xu, G., Fang, Q.Q., Keirans, J.E. and Durden, L.A. (2004) Ferritin gene coding sequences are conserved among eight hard tick species (Ixodida: Ixodidae). *Annals of Entomological Society of America* 97(3), 567–573. doi: 10.1603/0013-8746(2004)097[0567:FGCSAC]2.0.CO;2

Yan, F., Powell, D.R., Curtis, D.J. and Wong, N.C. (2020) From reads to insight: a hitchhiker's guide to ATAC-seq data analysis. *Genome Biology* 21, 22, doi: 10.1186/s13059-020-1929-3

You, M., Xuan, X., Tsuji, N., Kamio, T., Taylor, D.M., *et al.* (2003) Identification and molecular characterization of a chitinase from the hard tick *Haemaphysalis longicornis*. *Journal of Biological Chemistry* 278(10), 8556–8563. doi: 10.1074/jbc.M206831200

Zawadzki, J.L., Kotze, A.C., Fritz, J.A., Johnson, N.M., Hemsworth, J.E. *et al.* (2012) Silencing of essential genes by RNA interference in *Haemonchus contortus*. *Parasitology* 139(5), 613–629. doi: 10.1017/S0031182012000121

Zebrowski, G. (1926) Morphology of the American dog tick, *Dermacentor variabilis*. *American Entomological Society* 51, 331–369.

20   Silkworm Transgenesis and its Applications

Hideki Sezutsu[1],* and Toshiki Tamura[2]

[1]*National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan;* [2]*Silk Sciences and Technology Research Institute, Ami-machi, Inashiki-gun, Ibaraki, Japan*

20.1   Introduction

Silkworms (*Bombyx mori*) have been exploited for silk production for more than 5000 years. Global silk production reached 91,945 tons in 2020. Commercially available silk is predominantly produced by this insect. During the course of history, *B. mori* has been extensively domesticated. It is monophagous, feeds only on fresh mulberry leaves and cannot survive without human intervention; the larvae cannot obtain food in nature or escape from the rearing bed, and the moths cannot fly. These unusual characteristics significantly reduce the chances of survival of these genetically modified organisms if they were to be released into the natural environment.

Sericulture is an important industry in Asian and European countries. The genetics, physiology, biochemical processes and diseases of silkworms have been extensively studied. Research on the breeding of hybrid races, establishment of mass-rearing systems by artificial diets, and preservation of more than 450 stocks, including mutant, improved and geographical strains (Silkworm Base), have also been conducted. In addition, a genomic database for silkworms (KAIKObase, available at https://kaikobase.dna.affrc. go.jp; SilkDB, available at https://silkdb. bioinfotoolkits.net; both accessed 5 April 2022) has been established. Silkworms possess unique characteristics that facilitate the use of the species as a bioreactor; in fact, one larva has the ability to produce 0.2–0.5 g of protein.

The silkworm is also a useful research model for lepidopteran insects. The development of genetically modified silkworms will facilitate the elucidation of the function of lepidopteran insect genes, and the production of pharmaceuticals and biomaterials via the transgenic silkworm system. Furthermore, new silkworm races may be bred for sericulture. In this chapter, we introduce the recent progress, applications and future prospects of silkworm transgenesis.

20.2   Genetic Engineering of Silkworms

20.2.1   Construction of transgenic silkworms using transposons

The life cycle of a silkworm lasts for approximately 2 months (Fig. 20.1). Most strains are uni- or bivoltine and lay diapause eggs.

---

*Corresponding author, email: hsezutsu@naro.affrc.go.jp
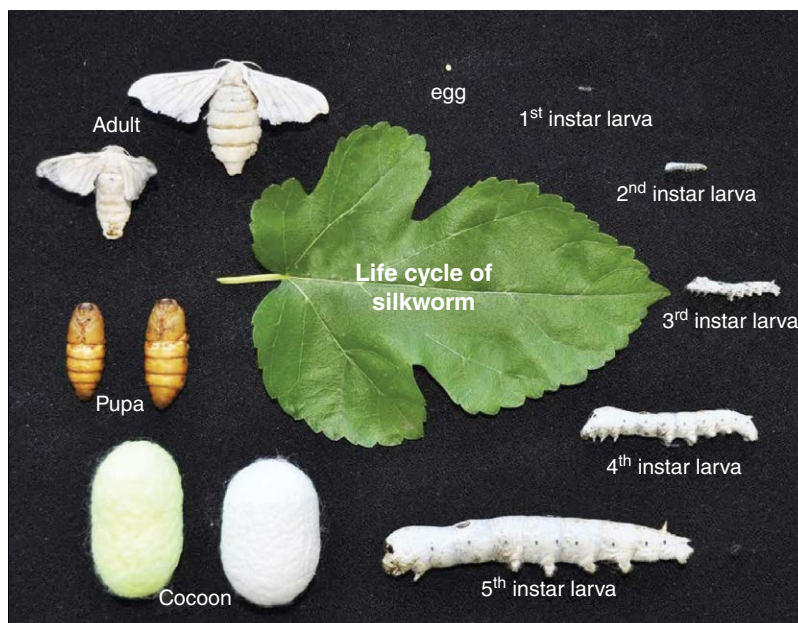
Fig. 20.1. Life cycle of a silkworm.

Embryonic development terminates 2 days after egg laying in the diapause eggs and an acid treatment is required to interrupt the termination. However, embryos of non-diapausing strains continue development and hatch approximately 10 days after oviposition. Therefore, non-diapausing strains are frequently used for generating transgenic silkworms. The w1-pnd strain is known to be ideal for genetic engineering. Its characteristic features, such as white eggs and eyes, facilitate the screening of transgenic insects and they become non-diapausing by the effect of the mutant gene, with pigmented and non-diapausing eggs (*pnd*). Nistari is another strain used for generating transgenic silkworms; it is non-diapausing and the chorion of the strain is softer than that in other strains. Therefore, penetration through the chorion with a glass needle for DNA injection is easier in Nistari than in other strains. Dazao also lays non-diapausing eggs and is used for generating transgenic silkworms.

The larval stage of a silkworm is of approximately 20 days when reared at 25–27°C. Silkworms can be reared on fresh mulberry leaves or an artificial diet. Non-diapausing strains tend to exhibit shorter lifespans than

diapausing silkworms. Standard silkworm races moult four times and the fully grown larva starts to spin silk to form the cocoon 20–25 days after hatching. To make a cocoon, the silkworm continues to spin silk fibre for 3 days and then becomes a pupa 5 days after spinning begins. The pupal stage lasts for approximately 10 days and then the moth emerges from the cocoon. Generally, eclosion occurs in the morning and the moths do not eat or drink; therefore, the survival duration of the moth is short. To obtain the next generation of silkworms, female and male moths are generally mated in the morning and separated in the afternoon. The separated females are then placed on egg-laying paper with a cover that discourages movement. The female moth generally lays eggs during the night. To create transgenic silkworms, it is important to inject DNA into the eggs within 8 h after egg laying, as the embryos reach the blastoderm stage at about 12 h after egg laying. If the DNA is injected later than this stage, it does not enter the nuclei (Tamura *et al.*, 1990). To obtain synchronized eggs, the mated moths are maintained at 25°C for 3–5 h and then in the refrigerator for 1–3 days. The synchronized

pre-blastoderm silkworm eggs can easily be obtained by using this system, as females start to lay eggs immediately after the moths are separated and transferred to a dark box at 24–26°C.

To generate transgenic silkworms, a solution containing transposase 'helper' and vector DNA is injected into the eggs at the pre-blastodermal stage. Hatched larvae are obtained and the eggs of the next generation are obtained from the emerging moths. The transgenic silkworms appear in the G_1 generation. Vectors containing the *piggyBac* or *Minos* transposons can be used to construct transgenic silkworms (Tamura *et al.*, 2000; Uchino *et al.*, 2007) (see O'Brochta, Chapter 1, this volume). The utilization of the *in vitro*-synthesized transposase mRNA in both transposons results in higher rates of transgenic insect generation compared with that produced upon injection of helper DNA. Generally, 20–50% of the moths grow from the injected eggs and 10–20% of them confer the transgene to their progeny. Injection of DNA into silkworm eggs requires a special method. Because the silkworm chorion is rather hard, the thin glass capillary breaks easily during the process of penetration into the eggs. Therefore, we use a special injector with a tungsten needle that makes a small hole in the eggs, and the capillary can be inserted into the eggs through the hole semi-automatically (Tamura *et al.*, 2007; Tatemastu *et al.*, 2012; Takasu *et al.*, 2014).

Many marker genes can be used to screen transgenic silkworms. The first transgenic silkworm was created using the enhanced green fluorescent protein (EGFP) marker under the control of the *Bombyx* cytoplasmic actin A3 gene promoter (Tamura *et al.*, 2000). The strong promoter activity directed gene expression in most tissues, and the transgenic silkworm larvae could be detected by the presence of green fluorescence throughout their bodies. An artificial promoter, *3xP3*, directs gene expression in the eye and nervous tissues of embryos (Horn *et al.*, 2000; Thomas *et al.*, 2002) (see Nolan and Hammond, Chapter 3, this volume). Various fluorescent protein genes under the control of this promoter can be used. The use of an artificial promoter is less labour-intensive since the screening can be done in the embryonic stage (Fig. 20.2). Other marker genes have also been developed and shown
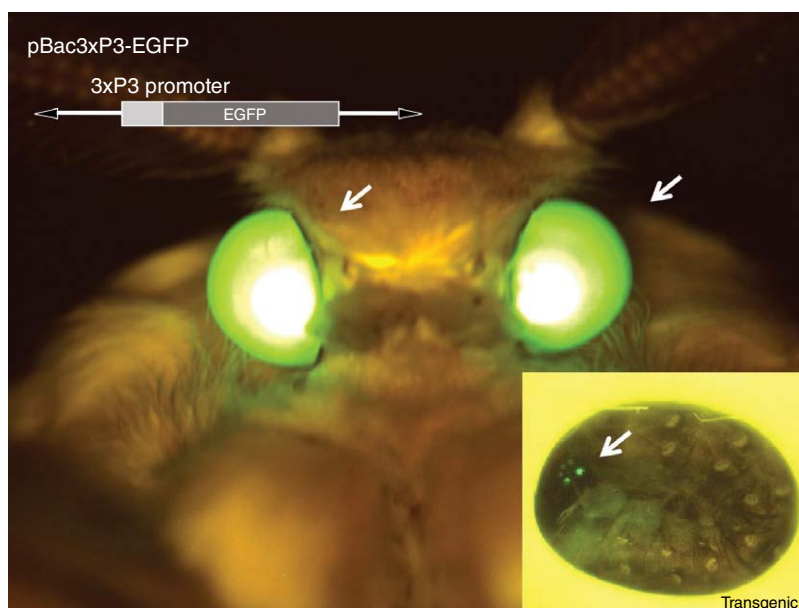
Fig. 20.2. Enhanced green fluorescent protein (EGFP) expression in the eyes of transgenic moths and embryos when the *3xP3 EGFP* marker gene was inserted into the genome. The white arrow indicates the eye expressing EGFP. A schematic structure of 3xP3EGF construct is shown in the white box and white arrow lines.

to be useful, including the *Bombyx* kynurenine 3-monooxygenase (*KMO*) gene directed by the *Bombyx* cytoplasmic actin A3 (*A3*) gene promoter (Kobayashi *et al.*, 2007) and arylalkylamine-*N*-acetyl transferase (NAT) with baculovirus immediate early 1 (*IE1*) gene promoter (Osanai-Futahashi *et al.*, 2012a). The expression of A3–KMO can be detected by the appearance of a brown larval integument at the first instar of the w-1 mutant strain. Expression of IE1-NAT changes the skin colour of newly hatched larvae from black to light brown.

For transgenesis of silkworms using transposons, the transgene is randomly inserted into the genome, which exerts a strong positional effect on expression (Uchino *et al.*, 2008). Further, the promoter activity is weaker than that of the endogenous gene. To circumvent these problems, gene insertion methods for integration into specific sites was studied (see Ahmed and Wimmer, Chapter 5, this volume). For this purpose, the use of FLP-recombinase and phiC31-integrase has been investigated. FLP-recombinase functions in the silkworm (Tomita *et al.*, 1999) and is specifically used for the excision of the transgene in the genome (Long *et al.*, 2012); however, successful integrations of transgenes have not been reported by this method. PhiC31-integrase, which catalyses the recombination between two target sequences attP and attB, may also be employed in silkworms (Yonemura *et al.*, 2012). Successful insertion of transgenes and recombinase-mediated cassette exchange has been reported by this method (Long *et al.*, 2013; Yonemura *et al.*, 2013; Yin *et al.*, 2014). In addition, an inducible expression system using the two genes has been reported (Wang *et al.*, 2021).

20.2.2 Genome editing and RNA interference

Genome editing was first demonstrated in silkworms using zinc-finger nuclease (ZFN), which recognizes the Bm*BLOS2* gene (Takasu *et al.*, 2010). ZFN is a chimeric enzyme that consists of a DNA recognition domain and a non-specific nuclease domain of the *Fok*I restriction enzyme. Bm*BLOS2* is one of the genes that controls the formation of uric acid granules in the larval epidermis. A mutation in this gene causes a translucent skin colour in the larval epidermis. When custom-designed Bm*BLOS2*-specific ZFN mRNA was injected into the embryos, several G_0 larvae exhibited translucent mosaic skin and germline mutants with translucent skin were observed in G_1 larvae. Gene analysis revealed that the mutation was caused by non-homologous end joining of the target gene. However, limitations of this method were that the mutagenesis rate varied with the designed ZFNs and the mutation frequency was low in most cases.

Genome editing methods using transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins (CRISPR/Cas9) are known to be more efficient methods. TALEN is a similar artificial enzyme that consists of a DNA-recognition domain and *Fok*I enzyme. The DNA-recognition domain is derived from the genus *Xanthomonas*. Injection of TALEN mRNAs into the embryo induces a mutation in the target gene with high efficiency (Ma *et al.*, 2012; Sajwan *et al.*, 2013). Several steps have been added to optimize the efficiency of mutagenesis (Takasu *et al.*, 2013). TALENs designed for a specific target sequence exhibited a mutation efficiency of more than 50%; in fact, most TALENs designed for target genes show a high capability of inducing mutations. Studies indicate that TALEN is more efficient than ZFN for targeted mutagenesis. TALEN is also used for site-specific transformation (Wang *et al.*, 2014a). For site-specific integration of target genes, TALEN is considered the most valuable tool in silkworms. Microhomology-mediated end-joining integration of transgenes (Nakade *et al.*, 2014; Tsubota and Sezutsu, 2017), genome editing using dsDNA and ssDNA donors (Takasu *et al.*, 2016), replacement of fibroin heavy-chain gene (Xu *et al.*, 2018) and targeted gene integration of the W chromosome (Zhang *et al.*, 2018) have been demonstrated in silkworms via this method.

Genome editing by CRISPR/Cas9 is a standard method to induce mutations and

to study the function of specific genes (see Concha and Papa, Chapter 7, this volume). CRISPR/Cas9 has been used more frequently compared with TALEN or ZFN because of its convenience of use. Editing of the silkworm genome by the CRISPR/Cas9 system was established in 2013 by injecting Cas9 mRNA and specific guide RNA into the eggs at the pre-blastodermal stage (Daimon *et al*., 2013; Wang *et al*., 2013a). Bm*BLOS2* was targeted in these experiments and somatic mutants with mosaic translucent and white-opaque skin colour in the epidermal cells was observed in G_0 larvae and the knockout mutants with translucent colour in the whole skin were obtained in G_1 larvae. Subsequently, this method was applied to other genes and was found to be effective for mutagenesis of target genes (Wei *et al*., 2014; Jia *et al*., 2018). Therefore, genome editing by CRISPR/Cas9 is the most frequently used method for inducing mutations in target genes and to study gene functions in silkworms. An injection of Cas9 protein and guide RNA into non-diapause eggs is now the most standard and popular method that is used in most experiments (Tomihara *et al*., 2021). In addition, transgenic silkworms harbouring the Cas9 gene were constructed (Ma *et al*., 2017a) and used for generating virus-resistant strains (Chen *et al*., 2017). The enzyme Cas12a (AsCpf1) is also valuable for genome editing in silkworms (Dong *et al*., 2020).

RNA interference (RNAi) involves gene silencing by double-stranded RNA (dsRNA) (see De Schutter and Smagghe, Chapter 4, this volume). RNAi is a powerful tool to silence specific genes in organisms. The effect of gene silencing is known to vary in insects and silencing by dsRNA injection has only limited effects in lepidopteran insects, including silkworms (Terenius *et al*., 2010). However, RNAi is efficient in embryos when dsRNA is injected into eggs at the pre-blastodermal stage (Quan *et al*., 2002; Liu *et al*., 2010). In some exceptional cases, dsRNA injected into larvae and pupa worked and was used to study the function of target genes. Hairpin RNAs expressed in specific tissues or cells of the transgenic silkworms function more effectively to silence the target gene. RNAi was initially implemented to inhibit the propagation of baculovirus in larvae and used for the breeding of *B. mori* nuclear polyhedrosis virus (BmNPV)-resistant strains (Yamada *et al*., 2002; Isobe *et al*., 2004; Kanginakudru *et al*., 2007; Subbaiah *et al*., 2013; Yang *et al*., 2017). The method was also used for the glycosylation of recombinant proteins produced by baculoviruses and inducible systems in silkworm (Dai *et al*., 2007). Certain factors, like sid-1, Argonaute2, BmREEPa and BmDicer2 that enhance the silencing effect, have been studied and the insertion and expression of them in silkworms were effective to increase the function of RNAi (Kobayashi *et al*., 2012; Li *et al*., 2015; Dong *et al*., 2017; You *et al*., 2020).

20.3 Applications of Gene Engineering in Functional Analyses

The gene engineering techniques discussed in Section 20.2 have been applied for the functional analyses of silkworm genes as it is a model lepidopteran insect (Xu and O'Brochta, 2015). The GAL4/UAS binary gene expression system was established in transgenic silkworms as a tool to study gene functions (Imamura *et al*., 2003) (see also Schetelig *et al*., Chapter 2, this volume). This system works efficiently in silkworms and has several advantages. First, the expression of the transgene in the silkworm is weaker than that of the endogenous gene; thus, the application of the binary expression system enhances its activity. Secondly, it facilitates the generation of transgenic silkworms, especially when the transgene exerts detrimental effects on the development of the silkworm. Because the gene is under UAS control, it is expressed only in the presence of GAL4; therefore, the gene inserted into the genome does not exhibit a detrimental effect. Thirdly, the GAL4 strains with different tissue and temporal specificities can be used for the expression of other genes. Enhancer trap lines based on the GAL4/UAS system have been constructed as an alternative method to control the expression of transgenes. These established lines are useful for controlling transgene expression in tissues in a

time-specific manner (Uchino *et al.*, 2008; Shimomura *et al.*, 2009). Additionally, several GAL4 variants enhance transgene expression and increase the expression of target genes (Kobayashi *et al.*, 2011; Liu *et al.*, 2019). A tetracycline-inducible transcription system has been developed to regulate the expression of transgenes (Tan *et al.*, 2013; Tatsuke *et al.*, 2013). Several tissue-specific promoters have been found to study the function of transgenes, such as the promoter that drives transgene expression in haemocyte oenocytoid cells (Tsubota *et al.*, 2013), midgut (Jiang *et al.*, 2013), a 2.9 kb upstream genomic fragment of hsp90 for all tissues (Tsubota *et al.*, 2014), testis (Xu *et al.*, 2014a), fat body (Deng *et al.*, 2013; Xu *et al.*, 2014b) and germline (Xu *et al.*, 2019a).

Kyushu University maintains 560 silkworm strains, most of which are mutants (available at: https://shigen.nig.ac.jp/silkwormbase, accessed April 2022). The National Agriculture and Food Research Organization in Japan preserves 540 silkworm races, including mutants, and geographical, improved, tropical and moult-character strains. Moreover, large numbers of silkworm strains are maintained in China, India, Korea, Italy and other countries, although some of them overlap. Several mutant genes have been identified and functionally characterized by genetic engineering (Fujii *et al.*, 2010; Daimon *et al.*, 2012). In particular, mutant genes causing morphological changes have been extensively studied. The genes responsible for cocoon colour have been investigated (Sakudoh *et al.*, 2007, 2010, 2013; Daimon *et al.*, 2010; Hirayama *et al.*, 2018). Several egg colour mutants like white egg-1 (*w-1*), white egg-2 (*w-2*), white egg-3 (*w-3*), red egg (*re*), pink-eyed white egg (*pe*) and brown egg 4 (*b4*) have been examined in detail and their mutated genes have been identified (Quan *et al.*, 2007; Tatematsu *et al.*, 2011; Osanai-Futahashi *et al.*, 2012b; 2015; Luo *et al.*, 2020; Tomihara *et al.*, 2021). Mutants with translucent skin colour are called oily silkworms. The ordinal silkworm possesses white and opaque skin, due to the accumulation of uric acid granules. These mutants are related to the formation of the granules and synthesis of uric acid in the epidermal cells

of silkworm larvae. To date, more than 20 loci related to translucent skin colour have been reported and the genes located on approximately half of the loci have been identified (Kiuchi *et al.*, 2011; Fujii *et al.*, 2012, 2020a,b; Wang *et al.*, 2013b; Zhang *et al.*, 2017a). Other mutant genes related to visible characters like albino, sex-linked chocolate colour, quail, and larval marking have also been studied (Liu *et al.*, 2010; Daimon *et al.*, 2012; Fujii *et al.*, 2013; Yoda *et al.*, 2014; Yuasa *et al.*, 2015). Genetically modified silkworms have been used for the study of physiology and behaviours. Functions of ecdysone and juvenile hormone (Tan *et al.*, 2005), behaviours related to the pheromone receptor (Yamagata *et al.*, 2008; Sakurai *et al.*, 2011, 2015), sex determination (Suzuki *et al.*, 2003; Kiuchi *et al.*, 2014), mechanisms of resistance to densovirus (Ito *et al.*, 2008), NPV, and Bt toxin (Atsumi *et al.*, 2012) have also been investigated.

20.4   Production of Recombinant Proteins for Pharmaceutical Use

The production of recombinant proteins by transgenic silkworms has been used for the creation of pharmaceutical proteins (Tomita, 2010, 2018; Xu, 2014; Itoh *et al.*, 2016, 2018; Sezutsu *et al.*, 2018). The organ used for the production of proteins in silkworms is the silk gland (Fig. 20.3). Recombinant protein production has been developed by genetic modification of the silk synthesis process. Silk consists of two types of proteins, called fibroin and sericin. Fibroin is the core protein forming the fibre, and sericin is a glue protein that covers the surface of the fibre and sticks two fibrils expelled from a pair of silk glands together. Fibroin is composed of three different proteins called fibroin heavy (H) and light (L) chains and fibrohexamarine (FHX). The formation of the complex comprising these three proteins enables the smooth secretion of fibroin molecules from the silk gland cells to the lumen (Inoue *et al.*, 2000). Sericin is the term for several different serine-rich proteins that are synthesized in the middle part of silk
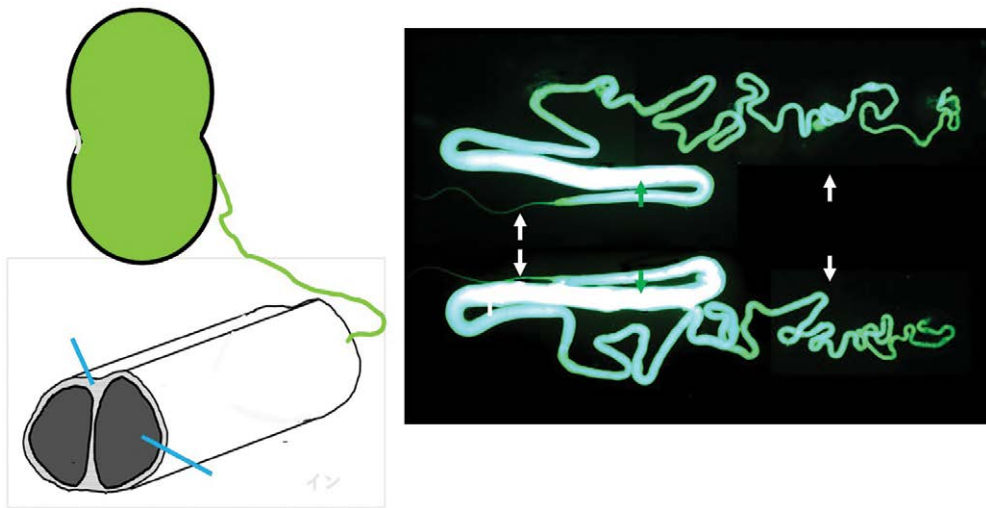
Fig. 20.3. A pair of silk glands synthesizing EGFP fused with fibroin at the 5th instar larva (right), and illustration of cocoon and silk filament structure (left). Silk proteins, fibroin and sericin, are synthesized in the posterior part and middle part of the silk glands (PSG and MSG), respectively. The cocoon is made from a long silk filament that contains a core fibroin filament covered with sericin. The silk is expelled from the anterior parts of the silk glands (ASG); the two fibroin filaments are merged and fused by the sericin layer that covers them.

gland (MSG). Three sericin genes (sericin-1, sericin-2 and sericin-3) encode for sericin proteins (Okamoto *et al.*, 1982; Garel *et al.*, 1997; Takasu *et al.*, 2007; Kludkiewicz *et al.*, 2009). Fibroin accounts for approximately 75% of all silk proteins and is produced in the posterior part of silk gland (PSG); the remaining 25% of silk proteins constitute sericin. As approximately 50% of the nutrients absorbed by mature silkworms is distributed to the silk proteins, it is a highly efficient protein production system.

Initially, the fibroin L-chain-encoding gene was used for the production of collagen or GFP as recombinant proteins (Tomita *et al.*, 2003; Inoue *et al.*, 2005). In these experiments, fusion proteins with the fibroin L-chain were constructed, the location of synthesis was confirmed to be the PSG, and thereafter the proteins were secreted into the lumen and expelled from the spinneret as silk. The promoter of the fibroin L-chain gene is efficient; however, the expression level of the transgene is less than 10% of that of the endogenous gene. The formation of a disulfide bond between the fusion gene product and the fibroin H-chain is indispensable

for secretion of the fusion protein from the PSG into the lumen. Recombinant protein production using the *FHX* gene for the production of DsRed has been reported (Royer *et al.*, 2005). The transgenic silkworms produced DsRed in PSG cells, secreted it into the lumen and exported it to the cocoon. The protein was spread over the whole silk fibre, suggesting that this system is adapted for the production of globular proteins in the PSG. Production using the H-chain gene has been also developed; the fused gene containing N- and C-terminal sequences of the fibroin H-chain contributes toward synthesis of the product, which is secreted into the cocoon of transgenic silkworms (Kojima *et al.*, 2007a; Kurihara *et al.*, 2007).

As the target proteins synthesized in the PSG are not secreted effectively unless they are fused with the H or L chain, an alternative production system using MSG cells has been studied. Two main systems have been developed and used for the production of recombinant proteins. The first system uses the *sericin-1* gene promoter, which directs protein expression in the MSG, including the baculovirus-enhancing

factors, *hr3* enhancer sequence and *IE1* transactivator (Tomita *et al*., 2007; Tomita, 2010; Ogawa *et al*., 2007). In this production system, *hr3* enhances the *sericin-1* gene promoter activity and *IE1* increases the activity of the enhancer. The GAL4/UAS system utilizes *GAL4*, which is under the control of the *sericin-1* gene promoter (Tatematsu *et al*., 2009). In this system, the transgenic silkworm containing *GAL4* and the target gene under the control of UAS was produced by crossing the independently constructed activator and effector lines. The protein produced in the MSG was efficiently secreted into the lumen and cocoons. The recombinant proteins produced in these systems were distributed in the surface with sericin and dissolved in aqueous solution. Therefore, the produced proteins can be easily extracted from the lumen of the MSG or cocoon without using denaturing chemicals. Mammalian-type signal peptide sequences are recognized in the MSG, and S-S bonds between the peptides can be automatically formed in the cells and the products are secreted into the lumen. In *N*-linked glycosylation, the recombinant proteins produced in the MSG possesses high mannose-, hybrid- and complex-type *N*-glycans and insect-specific paucimannose-type glycans were not present. In addition, they lack core alpha 1,3 fucosyl residues and possessed stronger antibody-dependent cell-mediated cytoxicity (ADCC) compared with the proteins synthesized by mammalian cells (Iizuka *et al*., 2009; Tada *et al*., 2015; Tomita, 2018). These systems were first applied to produce EGFP and human serum albumin (Ogawa *et al*., 2007; Tomita *et al*., 2007; Tatematsu *et al*., 2009). Subsequently, transgenic silkworms expressing H- and L-chain genes of the mouse antibody were found to efficiently synthesize IgG-type monoclonal antibodies in the MSG, which were secreted into the cocoon (Iizuka *et al*., 2009). The IgG-type antibody was composed of two H-chain molecules and two L-chain molecules. The IgG purified from the cocoons showed identical antigen-binding affinity compared with that of the native antibody hybridoma and contained high mannose- and complex-type *N*-glycans. A large amount of non-triple

helical collagen α1 chains was also produced using this system and the purified collagen was the same as that of denatured collagen (gelatin) (Adachi *et al*., 2010). This production system has been shown to be very useful for several proteins. For example, human–mouse chimeric anti-CD20 mAb produced by the transgenic silkworms exhibited a similar antigen-binding property and a stronger antibody-dependent cell-mediated cytoxicity compared with rituximab by Chinese hamster ovary cells (Tada *et al*., 2015; Aoyama *et al*., 2018). Feline interferon produced in transgenic silkworms was shown to exhibit a reduced risk of allergy as it lacked core alpha 1,3 fucosyl residues in its *N*-glycan chain (Minagawa *et al*., 2018). Human platelet-derived growth factor has also been produced in MSG, and the purified protein could promote the growth and proliferation of mouse cells (Chen *et al*., 2018). A human fibrinogen has been produced in the MSG, with an assembly identical to its native form. Human fibrinogen is a tetramer consisting of two alpha, two beta and two gamma chains. The recombinant fibrinogen automatically assembled and possessed native physical and coagulative properties (Minagawa *et al*., 2020). Numerous proteins, such as human lysosomal enzymes, tumour antigen proteins, lactoferrin with antibacterial and anti-inflammatory activities, and human vascular endothelial growth factor (rhVEGF), have been produced in transgenic silkworms (Xu, 2014; Itoh *et al*., 2016, 2018; Sezutsu *et al*., 2018; Tomita, 2018; Xu *et al*., 2019b; Zhang *et al*., 2019a; Kiyoshi *et al*., 2020; Yamada *et al*., 2021). To date, the maximum production yield of recombinant proteins from one larva is 15 mg. This is much smaller than that of the yield of one pair of silk glands, which can synthesize more than 500 mg of silk proteins. Commercialization of the recombinant proteins produced by transgenic silkworms has commenced. Test drugs for osteoporosis in humans and inflammation in dogs have been marketed by Nittobo Medical. Cosmetics using recombinant collagens, an amyloid detection kit containing antibodies and cell-culture base materials containing recombinant laminin have been marketed by Immuno-Biological Laboratories

Co. Ltd. Astellas Pharma Inc. and CURED Co. Ltd are developing pharmaceuticals for human use produced by transgenic silkworms at a pilot plant, in compliance with good manufacturing practices (GMP) (Tomita, 2018). In addition, some companies provide protein expression services using transgenic silkworms (Sezutsu *et al.*, 2018; Tomita, 2018). To improve the protein production system using genetically modified silkworms, it is important to develop a low-cost mass-rearing system that is compliant with GMP regulations and efficiently manages the transgenic strains. For this, the application of parthenogenic strains may be useful, because it enables the production of genetically identical populations and cryopreservation of the germ plasm of transgenic silkworms (Grenier *et al.*, 2004; Zabelina *et al.*, 2015a,b, 2021). To increase the production of recombinant proteins, the strains with low expression or a knockout of silk-producing genes has been shown to be effective (Ma *et al.*, 2017b; Wu *et al.*, 2021). Furthermore, substitutions of the initiation codon context motifs and replacement of endogenous silk genes with target genes by homologous recombination have been performed to increase productivity (Tatematsu *et al.*, 2014; Xu *et al.*, 2018).

20.5 Construction of Modified Silk and its Possible Use as a Biomaterial

Natural silk can be modified by using fibroin H-chain- and fibroin L-chain-encoding genes. Silk modification was performed for the first time using the fibroin L-chain vector, in which the fibroin L-chain was fused with the green fluorescent protein (GFP*)* gene (Tomita *et al.*, 2003; Inoue *et al.*, 2005). Transgenic silkworms harbouring the fluorescent gene produced GFP-containing silks. Subsequently, modified silk possessing the human fibroblast growth factor was synthesized by the same method, indicating that silk is a valuable biomaterial for tissue engineering (Hino *et al.*, 2006). In a similar experiment, a silkworm strain producing a fibroin L-chain fused with partial collagen or fibronectin

peptide sequences was generated, and a film was made from the modified silk. Characterization of the modified silk showed that it exhibited a higher cell-adhesive activity than the original unmodified silk (Yanagisawa *et al.*, 2007). The proportion of fused fibroin L-chain in the modified silk could be increased using the transgenic *Nd-s* mutant silkworm. The vector made from the fibroin H-chain-encoding gene has been used more frequently. The fibroin H-chain is a large protein with a molecular weight of 350–400 kDa, which consists of N- and C-terminal domains and the core region including the (Gly–Ser–Gly–Ala–Gly–Ala)$_n$ repeat sequences, which determines the characteristics of the silk fibre (Zhou *et al.*, 2000). This gene was examined in one of the earlier studies involving molecular biology techniques (Suzuki and Brown, 1972; Suzuki *et al.*, 1972). The promoter of this gene has been well characterized using *in vitro* transcription assays in cell-free extracts (Suzuki *et al.*, 1986; Hui and Suzuki, 1995). The vector contains the promoter region and the N- and C-terminal domains for the secretion of the fusion protein (Kojima *et al.*, 2007a; Kurihara *et al.*, 2007). To produce the desired modified silk, the target sequence is inserted between the N- and C-terminal domains. When the EGFP gene was inserted into the vector, the transgenic silkworm produced silk with strong green fluorescence colour (Kojima *et al.*, 2007a). However, the proportion of the fusion protein that occupied the entire silk fibre was low; most of the silk proteins were composed of natural fibroin molecules synthesized from the original endogenous fibroin H-chain-encoding gene.

Many different types of modified silk have been constructed using these systems. The constructed silks can be categorized into two groups on the basis of the purpose of utilization. The first one is for making textiles and the other is for use as biomaterials for medical applications. The fluorescent-colour silks, spider silks and antibacterial silks are mainly employed in textiles. The second group comprises silks containing peptides or protein sequences that enhance their affinity toward living cells.

Silks with different fluorescent colours have been synthesized and utilized in different types of textiles (Iizuka *et al*., 2013; Iizuka, 2016; Shimizu, 2018). To date, several silks with different colours, such as green, red, orange, or blue, have been synthesized. Silks with very thin fibres have also been produced and used for making special clothing. Silks incorporating peptides that can change their stainability have also been created and utilized for making specialized clothing items (Iizuka, 2016). These silks have been shown to have commercial value, and the behavioural and physiological characteristics of the transgenic silkworms that produce these modified silks are identical to those of ordinary silkworms. A large number of larvae can be reared in a rearing house using fresh mulberry leaves, and their cocoons can be harvested. The mechanical characteristics of modified silks are similar to those of non-recombinant natural silk, therefore large-scale reeling of modified silk and producing fabrics from it is possible (Iizuka *et al*., 2013). Japanese garments made from different fluorescent silks of green, red, or orange colour are shown in Fig. 20.4. Field rearing of transgenic silkworms that produce these silks has started in Japan (Fig. 20.5). Different strains of silkworms have been generated that synthesize silk containing spider proteins, which can be expelled (Kojima *et al*.,

2007b; Zhang *et al*., 2008, 2019b; Wen *et al*., 2010; Teule *et al*., 2012; Kuwana *et al*., 2014; You *et al*., 2018). The silk thread made by the transgenic silkworms was stronger than that

Fig. 20.4. A Japanese kimono made from fluorescent silks. This garment displays the different fluorescent colours upon observation through a yellow filter under a blue LED light.

Fig. 20.5. Rearing of transgenic silkworms by a farmer.

of natural silk. To increase the expression of the target gene, the endogenous fibroin gene was substituted by genome editing, facilitating the production of spider silk in silkworms (Xu *et al*., 2018). Silks with antimicrobial activity have been synthesized, as they contain antimicrobial peptides, cecropin, moricin and Gloverin2 (Li *et al*., 2014; Saviane *et al*., 2018; Wang *et al*., 2019a). Furthermore, silks with additional polyalanine residues have been reported to exhibit excellent mechanical properties (Zhao *et al*., 2021).

Silks containing RGD amino acid sequences, growth factors, human collagen, single-chain variable fragment (scFv) and azide amino acids can be utilized as biomaterials (Kambe *et al*., 2011, 2015; Sato *et al*., 2012, 2014, 2017; Asakura *et al*., 2014, 2019; Wang *et al*., 2014b; Teramoto and Kojima, 2014; Teramoto *et al*., 2016, 2018, 2019, 2020; Li *et al*., 2014; Saotome *et al*., 2015; Woltje *et al*., 2018; Baba *et al*., 2018). They can be used for coating the surface of plates used for cell culture, for manufacturing small-diameter silk vascular grafts, artificial skins to heal wounds, and sponges for bone construction.

20.6   Utilization of Genetically Modified Silkworms in Sericulture

After the methods of transgenesis were established, the different applications of genetically modified silkworms in sericulture were explored. Several studies indicate the possibility of increased silk production in genetically modified silkworms. Overexpression of certain genes that were introduced into the genome increased silk production. *Ras* oncogene regulates cell growth and protein synthesis. Ectopic expression of Ras in silk glands resulted in increased silk production (Ma *et al*., 2011). The overexpression of Yorkie (CA), BmGT1-L, and Myc in silk glands also increased the silk yield in transgenic silkworms (Zhang *et al*., 2017b; Tang *et al*., 2020; Qian *et al*., 2021). Ectopic expression of the ecdysteroid *UDP* glucosyltransferase-encoding gene of baculovirus reduced the ecdysone titre in the haemolymph of the silkworm larvae and contributed towards an increased shell ratio of cocoons (Shen *et al*., 2018). However, further studies are required to determine the utility of these genes for generating new strains. Genetic sexing is also useful to increase the production yield. The productivity of the silk is high in males compared with females; therefore, the construction of a male rearing system for transgenic silkworms has been explored (Tan *et al*., 2013; Xu *et al*. 2014b; Zhang *et al*., 2018).

The application of transgenic silkworms in breeding has focused on disease-resistant strains, particularly against *B. mori* nuclear polyhedrosis virus (BmNPV). BmNPV is a serious disease of silkworms and has a significant impact on silk production; moreover, no resistant strain has been found in the silkworm strains. Therefore, a resistant strain has not been established by the ordinal hybridization breeding method, although considerable efforts have been made by breeders. Genetic engineering may provide an opportunity to generate virus-resistant silkworms (Jiang and Xia, 2014; Jiang, 2021). This may be done by knocking down virus transcripts by RNA interference (Yamada *et al*., 2002; Isobe *et al*., 2004; Kanginakudru *et al*., 2007; Jiang *et al*., 2012; Subbaiah *et al*., 2013; Zhang *et al*., 2013) (see Franz, Chapter 22, this volume). The major genes associated with the propagation of BmNPV were selected and corresponding short hairpin mRNA specific to the viral genes were synthesized. The hairpin RNA functions as dsRNA and degrades the viral transcripts and inhibits propagation. Several transgenic silkworms were conferred with resistance to viruses by this technique. These silkworms were reared in farms in India and their tendency for infections was evaluated. Other attempts to construct NPV-resistant strains were also made. For example, transgenic silkworms expressing antiviral proteins (Jiang *et al*., 2012), applied in the CRISPR/Cas9 mediated genome editing to destroy the virus, were generated and were demonstrated to exhibit enhanced resistance to BmNPV (Dong *et al*., 2018a,b, 2020). To utilize the resistant strains in the field, further studies related to the generation of high-resistance strains, breeding and safety assessment are warranted. Strains

resistant to other pathogens have also been studied. A gene encoding an amino acid transporter was shown to cause resistance to *Bombyx parvo*-like virus (Ito *et al.*, 2008). Silkworms with antiviral activities against densovirus and cytoplasmic polyhedrosis virus were generated in several studies (Jiang *et al.*, 2017; Sun *et al.*, 2018; Zhao *et al.*, 2018). Silkworms exhibiting resistance against entomopathogenic bacteria and *Nosema bombycis* have also been reported (Huang *et al.*, 2018; Xu *et al.*, 2020; Dong *et al.*, 2021).

20.7 Future Prospects

Transgenic silkworms have been utilized to study gene functions, to produce recombinant proteins and modified recombinant silks, to improve breeding techniques for high silk yields and to provide resistance to diseases. Further studies are required to expand the applications of transgenic silkworms. Although homologous recombination and targeted insertion of transgenes using TALEN have been successful (Nakade *et al.*, 2014; Xu *et al.*, 2018; Zhang *et al.*, 2018), it cannot be designated as a standard method. A yield of 10 mg of recombinant protein per silkworm larva has been reported (Adachi *et al.*, 2010) but this yield has not been enhanced further. It is essential to develop cost-effective production systems for recombinant proteins to facilitate commercialization. Mass-rearing systems that can facilitate the production of pharmaceuticals need to be developed. Silkworm rearing machines using artificial diets have proven to be effective for mass-rearing of transgenic silkworms and enable the commercialization of products (Sezutsu *et al.*, 2018; Tomita, 2018). Another important point of concern is the safety assessment of genetically modified silkworms reared in farms. It has been reported that transgenic silkworms released in the field did not produce toxic substances and were killed by birds and ants (Komoto *et al.*, 2014). Similarly, genetically modified silkworms were shown to be safe and did not contain toxic substances (Jiang *et al.*, 2021b). Furthermore, the recombinant DNA harboured by the transgenic silkworms did not transfer to chickens even when they were present in the chicken feed (Wang *et al.*, 2019b). Another major concern is the possibility of hybridization of engineered silkworms with wild silkworms, particularly *Bombyx mandarina*, which is spread in the mulberry fields of Asian countries. However, no hybridization between domesticated and wild silkworms was observed in the field populations around sericulture farms, indicating that the transfer of recombinant DNA from the transgenic silkworms to the field organisms does not occur in the natural environment (Yukuhiro *et al.*, 2013; Komoto *et al.*, 2016; Komoto and Tomita, 2020). Similar data pertaining to the safety of field rearing of transgenic silkworms has been obtained by several researchers.

References

Adachi, T., Wang, X., Murata, T., Obara, M., Akutsu, H. *et al.* (2010) Production of a non-triple helical collagen alpha chain in transgenic silkworms and its evaluation as a gelatin substitute for cell culture. *Biotechnology and Bioengeering* 106(6), 860–870.

Aoyama, M., Tada, M., Tatematsu, K.I., Hashii, N., Sezutsu, H. and Ishii-Watabe, A. (2018) Effects of amino acid substitutions on the biological activity of anti-CD20 monoclonal antibody produced by transgenic silkworms (Bombyx mori). *Biochemical and Biophysical Research Communications* 503(4), 2633–2638.

Asakura, T., Isozaki, M., Saotome, T., Tatematsu, K.I., Sezutsu, H. *et al.* (2014) Recombinant silk fibroin incorporated cell-adhesive sequences produced by transgenic silkworm as a possible candidate for use in vascular graft. *Journal of Materials Chemistry B* 2(42), 7375–7383.

Asakura, T., Tanaka, T. and Tanaka, R. (2019) Advanced silk fibroin biomaterials and application to small-diameter silk vascular grafts. *ACS Biomaterials Science & Engineering* 5(11), 5561–5577.

Atsumi, S., Miyamoto, K., Yamamoto, K., Narukawa, J., Kawai, S. *et al.* (2012) Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, *Bombyx mori*. *Proceedings of the National Academy of Sciences USA* 109(25), E1591–E1598.

Baba, A., Matsushita, S., Kitayama, K., Asakura, T., Sezutsu, H. *et al.* (2018) Silk fibroin produced by transgenic silkworms overexpressing the Arg-Gly-Asp motif accelerates cutaneous wound healing in mice. *Journal of Biomedical Materials Research Part B Applied Biomaterials* 107(1), 97–103.

Chen, S., Hou, C., Bi, H., Wang, Y., Xu, J. *et al.* (2017) Transgenic clustered regularly interspaced short palindromic repeat/Cas9-mediated viral gene targeting for antiviral therapy of *Bombyx mori* nucleopolyhedrovirus. *Journal of Virology* 91(8), e02465-16.

Chen, W., Wang, F., Tian, C., Wang, Y., Xu, S. *et al.* (2018) Transgenic silkworm-based silk gland bioreactor for large scale production of bioactive human platelet-derived growth factor (PDGF-BB) in silk cocoons. *International Journal of Molecular Science* 19(9), 2533.

Dai, H., Jiang, R., Wang, J., Xu, G., Cao, M. *et al.* (2007) Development of a heat shock inducible and inheritable RNAi system in silkworm. *Biomolecular Engineering* 24(6), 625–630.

Daimon, T., Hirayama, C., Kanai, M., Ruike, Y., Meng, Y. *et al.* (2010) The silkworm Green b locus encodes a quercetin 5-O-glucosyltransferase that produces green cocoons with UV-shielding properties. *Proceedings of the National Academy of Sciences USA* 107(25), 11471–11476.

Daimon, T., Kozaki, T., Niwa, R., Kobayashi, I., Furuta, K. *et al.* (2012) Precocious metamorphosis in the juvenile hormone-deficient mutant of the silkworm, *Bombyx mori*. *PLoS Genetics* 8(3), e1002486.

Daimon, T., Kiuchi, T. and Takasu, Y. (2013) Recent progress in genome engineering techniques in the silkworm, *Bombyx mori*. *Development, Growth, & Differentiation* 56(1), 14–25.

Deng, D., Xu, H., Wang, F., Duan, X., Ma, S. *et al.* (2013) The promoter of Bmlp3 gene can direct fat body-specific expression in the transgenic silkworm, *Bombyx mori*. *Transgenic Research* 22(5), 1055–1063.

Dong, X.L., Liu, T.H., Wang, W., Pan, C.X., Du, G.Y. *et al.* (2017) Transgenic RNAi of BmREEPa in silkworms can enhance the resistance of silkworm to *Bombyx mori* nucleopolyhedrovirus. *Biochemical and Biophysical Research Communications* 483(2), 855–859.

Dong, Z., Hu, Z., Qin, Q., Dong, F., Huang, L. *et al.* (2018a) CRISPR/Cas9-mediated disruption of the immediate early-0 and 2 as a therapeutic approach to *Bombyx mori* nucleopolyhedrovirus in transgenic silkworm. *Insect Molecular Biology* 28(1), 112–122.

Dong, Z., Huang, L., Dong, F., Hu, Z., Qin, Q. *et al.* (2018b) Establishment of a baculovirus-inducible CRISPR/Cas9 system for antiviral research in transgenic silkworms. *Applied Microbiology and Biotechnology* 102(21), 9255–9265.

Dong, Z., Qin, Q., Hu, Z., Zhang, X., Miao, J. *et al.* (2020) CRISPR/Cas12a mediated genome editing enhances *Bombyx mori* resistance to BmNPV. *Frontiers in Bioengineering and Biotechnology* 8, 841.

Dong, Z., Zheng, N., Hu, C., Huang, X., Chen, P. *et al.* (2021) Genetic bioengineering of overexpressed guanylate binding protein family BmAtlastin-n enhances silkworm resistance to *Nosema bombycis*. *International Journal of Biological Macromolecules* 172, 223–230.

Fujii, T., Daimon, T., Uchino, K., Banno, Y., Katsuma, S. *et al.* (2010) Transgenic analysis of the BmBLOS2 gene that governs the translucency of the larval integument of the silkworm, *Bombyx mori*. *Insect Molecular Biology* 19(5), 659–667.

Fujii, T., Banno, Y., Abe, H., Katsuma, S. and Shimada, T. (2012) A homolog of the human Hermansky-Pudluck syndrome-5 (HPS5) gene is responsible for the oa larval translucent mutants in the silkworm, *Bombyx mori*. *Genetica* 140(10-12), 463–468.

Fujii, T., Abe, H., Kawamoto, M., Katsuma, S., Banno, Y and Shimada, T. (2013) Albino (al) is a tetrahydrobiopterin (BH4)-deficient mutant of the silkworm *Bombyx mori*. *Insect Biochemistry and Molecular Biology* 43(7), 594–600.

Fujii, T., Kakino, K., Tanaka, M., Lee, J.M., Kusakabe, T. and Banno, Y. (2020a) A defect in purine nucleotide metabolism in the silkworm, *Bombyx mori*, causes a translucent larval integument and male infertility. *Insect Biochemistry and Molecular Biology* 126, 103458.

Fujii, T., Yamamoto, K. and Banno, Y. (2020b) Molybdenum cofactor deficiency causes translucent integument, male-biased lethality, and flaccid paralysis in the silkworm *Bombyx mori* [Yamamoto translucent (oya)]. *Insect Biochemistry and Molecular Biology* 73, 20–26.

Garel, A., Deleage, G. and Prudhomme, J.-C. (1997) Structure and organization of the *Bombyx mori* sericin 1 gene and of the sericins 1 deduced from the sequence of the Ser 1B cDNA. *Insect Biochemistry and Molecular Biology* 27(5), 469–477.

Grenier, A.M., Da Rocha, M., Jalabert, A., Royer, C., Mauchamp, B. and Chavancy, G. (2004) Artificial parthenogenesis and control of voltinism to manage transgenic populations in *Bombyx mori*. *Journal of Insect Physiology* 50(8), 751–760.

Hino, R., Tomita, M. and Yoshizato K.L. (2006) The generation of germline transgenic silkworms for the production of biologically active recombinant fusion proteins of fibroin and human basic fibroblast growth factor. *Biomaterials* 27(33), 5715–5724.

Hirayama, C., Mase, K., Iizuka, T., Takasu, Y., Okada, E. and Yamamoto, K. (2018) Deficiency of a pyrroline-5-carboxylate reductase produces the yellowish green cocoon 'Ryokuken' of the silkworm, *Bombyx mori*. *Heredity (Edinburgh)* 120(5), 422–436.

Horn, C., Jaunich, B. and Wimmer, E.A. (2000) Highly sensitive, fluorescent transformation marker for *Drosophila* transgenesis. *Development Genes and Evolution* 210(12), 623–629.

Huang, Y., Chen, J., Sun, B., Zheng, R., Li, B. *et al.* (2018) Engineered resistance to *Nosema bombycis* by in vitro expression of a single-chain antibody in Sf9-III cells. *PLoS ONE* 13(2), e0193065.

Hui, C.C. and Suzuki, Y. (1995) Regulation of the silk protein genes and the homeobox genes in silk gland development. In: Goldsmith, M.R. and Wilkins, A.S. (eds) *Molecular Model Systems in the Lepidoptera*. Cambridge University Press, Cambridge, UK, pp. 249–271.

Iizuka, M., Ogawa, S., Takeuchi, A., Nakakita, S., Kubo, Y. *et al.* (2009) Production of a recombinant mouse monoclonal antibody in transgenic silkworm cocoons. *FEBS Journal* 276(20), 5806–5820.

Iizuka, T. (2016) Development of commercial transgenic silkworms producing high-performance silks. *Breeding Research* 18, 158–164.

Iizuka, T., Sezutsu, H., Tatematsu, K., Kobayashi, I., Yonemura, N. *et al.* (2013) Colored fluorescent silk made by transgenic silkworms. *Advanced Functional Materials* 23(42), 5232–5239.

Imamura, M., Nakai, J., Inoue, S., Quan, G.X., Kanda, T and Tamura, T. (2003) Targeted gene expression using the GAL4/UAS system in the silkworm *Bombyx mori*. *Genetics* 165(3), 1329–1340.

Inoue, S., Kanda, T., Imamura, M., Quan, G.X., Kojima, K. *et al.* (2005) A fibroin secretion-deficient silkworm mutant, Nd-sD, provides an efficient system for producing recombinant proteins. *Insect Biochemistry and Molecular Biology* 35(1), 51–59.

Inoue, S., Tanaka, K., Arisaka, F., Kimura, S., Ohtomo, K. and Mizuno, S. (2000) Silk fibroin of *Bombyx mori* is secreted, assembling a high molecular mass elementary unit consisting of H-chain, L-chain, and P25, with a 6:6:1 molar ratio. *Journal of Biological Chemistry* 275(51), 40517–40528.

Isobe, R., Kojima, K., Matsuyama, T., Quan, G.X., Kanda, T. *et al.* (2004) Use of RNAi technology to confer enhanced resistance to BmNPV on transgenic silkworms. *Archives of Virology* 149(10), 1931–1940.

Ito, K., Kidokoro, K., Sezutsu, H., Nohata, J., Yamamoto, K. *et al.* (2008) Deletion of a gene encoding an amino acid transporter in the midgut membrane causes resistance to a *Bombyx parvo*-like virus. *Proceedings of the National Academy of Sciences USA* 105(21), 7523–7527.

Itoh, K., Kobayashi, I., Nishioka, S., Sezutsu, H., Machii, H and Tamura, T. (2016) Recent progress in development of transgenic silkworms overexpressing recombinant human proteins with therapeutic potential in silk glands. *Drug Discovery and Therapeutics* 10(1), 34–39.

Itoh, K., Nishioka, S.I., Hidaka, T., Tsuji, D. and Maita, N. (2018) [Development of enzyme drugs derived from transgenic silkworms to treat lysosomal diseases]. *Yakugaku Zasshi* 138(7), 885–893.

Jia, L., Ma, S.Y., Zhang, T., Xing, W.Q., Liu, Y. *et al.* (2018) Enhanced *Bombyx* genome editing via Cas9 ribonucleoprotein injection. *Insect Science* 26(6), 1059–1062.

Jiang, L. (2021) Insights into the antiviral pathways of the silkworm *Bombyx mori*. *Frontiers in Immunology* 12, 639092.

Jiang L. and Xia, Q. (2014) The progress and future of enhancing antiviral capacity by transgenic technology in the silkworm *Bombyx mori*. *Insect Biochemistry and Molecular Biology* 48, 1–7.

Jiang, L., Wang, G., Cheng, T., Yang, Q., Jin, S. *et al.* (2012) Resistance to *Bombyx mori* nucleopolyhedrovirus via overexpression of an endogenous antiviral gene in transgenic silkworms. *Archives of Virology* 157(7), 1323–1328.

Jiang, L., Cheng, T., Dang, Y., Peng, Z., Zhao, P. *et al.* (2013) Identification of a midgut-specific promoter in the silkworm *Bombyx mori*. *Biochemical and Biophysical Research Communications* 433(4), 542–546.

Jiang, L., Peng, Z., Guo, H., Sun, J., Sun, Q. *et al.* (2017) Enhancement of antiviral capacity of transgenic silkworms against cytoplasmic polyhedrosis virus via knockdown of multiple viral genes. *Developmental & Comparative Immunology* 77, 138–140.

Jiang, L., Goldsmith, M.R. and Xia, Q. (2021a) Advances in the arms race between silkworm and baculovirus. *Frontiers in Immunology* 12(30), 628151.

Jiang, L., Wang, Y., Guo, H., Sun, Q., Xie, E. *et al.* (2021b) Toxicological evaluation of transgenic silkworms. *Toxicology Research (Cambridge)* 9(6), 845–853.

Kambe, Y., Takeda, Y., Yamamoto, K., Kojima, K., Tamada, Y. and Tomita, N. (2011) Effect of RGDS-expressing fibroin dose on initial adhesive force of a single chondrocyte. *Bio-Medical Materials and Engineering* 20(6), 309–316.

Kambe, Y., Kojima, K., Tamada, Y., Tomita, N. and Kameda, T. (2015) Silk fibroin sponges with cell growth-promoting activity induced by genetically fused basic fibroblast growth factor. *Journal of Biomedical Materials Research Part A* 104(1), 82–93.

Kanginakudru, S., Royer, C., Edupalli, S.V., Jalabert, A., Mauchamp, B. *et al.* (2007) Targeting ie-1 gene by RNAi induces baculoviral resistance in lepidopteran cell lines and in transgenic silkworms. *Insect Molecular Biology* 16(5), 635–644.

Kiuchi, T., Banno, Y., Katsuma, S. and Shimada, T. (2011) Mutations in an amino acid transporter gene are responsible for sex-linked translucent larval skin of the silkworm, Bombyx mori. *Insect Biochemistry and Molecular Biology* 41(9), 680–687.

Kiuchi, T., Koga, H., Kawamoto, M., Shoji, K., Sakai, H. *et al.* (2014) A single female-specific piRNA is the primary determiner of sex in the silkworm. *Nature* 509(7502), 633–636.

Kiyoshi, M., Tatematsu, K.I., Tada, M., Sezutsu, H., Shibata, H. and Ishii-Watabe, A. (2020) Structural insight and stability of TNFR-Fc fusion protein (Etanercept) produced by using transgenic silkworms. *Journal of Biochemistry* 169(1), 25–33.

Kludkiewicz, B., Takasu, Y., Fedic, R., Tamura, T., Sehnal, F. and Zurovec, M. (2009) Structure and expression of the silk adhesive protein Ser2 in Bombyx mori. *Insect Biochemistry and Molecular Biology* 39(12), 938–946.

Kobayashi, I., Uchino, K., Sezutsu, H., Iizuka, T. and Tamura, T. (2007) Development of a new *piggyBac* vector for generating transgenic silkworms using the kynurenine 3-mono oxygenase gene. *Journal of Insect Biotechnology and Sericology* 76(3), 145–148.

Kobayashi, I., Kojima, K., Uchino, K., Sezutsu, H., Iizuka, T. *et al.* (2011) An efficient binary system for gene expression in the silkworm, *Bombyx mori*, using GAL4 variants. *Archives of Insect Biochemistry and Physiology* 76(4), 195–210.

Kobayashi, I., Tsukioka, H., Komoto, N., Uchino, K., Sezutsu, H. *et al.* (2012) SID-1 protein of *Caenorhabditis elegans* mediates uptake of dsRNA into Bombyx cells. *Insect Biochemistry and Molecular Biology* 42(2), 148–154.

Kojima, K., Kuwana, Y., Sezutsu, H., Kobayashi, I., Uchino, K, *et al.* (2007a) A new method for the modification of fibroin heavy chain protein in the transgenic silkworm. *Bioscience, Biotechnology, and Biochemistry* 71(12), 2943–2951.

Kojima, K., Kuwana, Y. and Sezutsu, H. (2007b) NMR analysis of silk produced by transgenic silkworm which expresses spider fiber protein in silk. *Kobunnshi Ronbunshu [Japanese Journal of Polymer Science and Technology]* 64, 817–819.

Kômoto, N. and Tomita, S. (2020) Risk assessment of transgenic silkworms. In: Chaurasia, A., Hawksworth, D.L. and Pessoa de Miranda, M. (eds) *GMOs. Implications for Biodiversity Conservation and Ecological Processes*. Series: Topics in Biodiversity Conservation 19. Springer, Cham, Switzerland, pp. 233–240.

Kômoto, N., Kuwabara, N. and Yukuhiro, K. (2016) Absence of hybrids between the domesticated silkmoth, *Bombyx mori*, and the wild mulberry silkmoth, *B. mandarina*, in natural populations around sericulture farms. *Journal of Insect Biotechnology and Sericology* 85(3), 67–81.

Kômoto, N., Tsuda, M., Okada, E., Iizuka, T., Kuwabara, N. *et al.* (2014) Development of methods for risk assessment of transgenic silkworms rearing on biodiversity. *Sanshi-Konchu Biotech* 83(2), 171–179.

Kurihara, H., Sezutsu, H., Tamura, T. and Yamada, K. (2007) Production of an active feline interferon in the cocoon of transgenic silkworms using the fibroin H-chain expression system. *Biochemical and Biophysical Research Communications* 355(4), 976–980.

Kuwana, Y., Sezutsu, H., Nakajima, K., Tamada, Y. and Kojima, K. (2014) High-toughness silk produced by a transgenic silkworm expressing spider (*Araneus ventricosus*) dragline silk protein. *PLoS ONE* 9(8), e105325.

Li, Z., Jiang, Y., Cao, G., Li, J., Xue, R. and Gong, C. (2014) Construction of transgenic silkworm spinning antibacterial silk with fluorescence. *Molecular Biology Reports* 42(1), 19–25.

Li, Z., Zeng, B., Ling, L., Xu, J., You, L. *et al.* (2015) Enhancement of larval RNAi efficiency by over-expressing Argonaute2 in *Bombyx mori*. *International Journal of Biological Sciences* 11(2), 176–185.

Liu, C., Yamamoto, K., Cheng, T.C., Kadono-Okuda, K., Narukawa, J. *et al.* (2010) Repression of tyrosine hydroxylase is responsible for the sex-linked chocolate mutation of the silkworm, *Bombyx mori*. *Proceedings of the National Academy of Sciences USA* 107(29), 12980–12985.

Liu, R., Zeng, W., Tan, T., Chen, T., Luo, Q. *et al.* (2019) Insights into the regulatory characteristics of silkworm fibroin gene promoters using a modified Gal4/UAS system. *Transgenic Research* 28(5-6), 627–636.

Long, D.P., Zhao, A.C., Chen, X.J., Zhang, Y., Lu, W.J. *et al.* (2012) FLP recombinase-mediated site-specific recombination in silkworm, *Bombyx mori*. *PLoS ONE* 7(6), e40150.

Long, D., Zhao, A., Xu, L., Lu, W., Guo, Q. *et al.* (2013) In vivo site-specific integration of transgene in silkworm via PhiC31 integrase-mediated cassette exchange. *Insect Biochemistry and Molecular Biology* 43(11), 997–1008.

Luo, J.W., An, E.X., Lu, Y.R., Yang, L., Gai, T.T. *et al.* (2020) Molecular basis of the silkworm mutant re(l) causing red egg color and embryonic death. *Insect Science* 28(5), 1290–1299.

Ma, L., Xu, H., Zhu, J., Ma, S., Liu, Y. *et al.* (2011) Ras1(CA) overexpression in the posterior silk gland improves silk yield. *Cell Research* 21(6), 934–943.

Ma, S., Zhang, S., Wang, F., Liu, Y., Xu, H. *et al.* (2012) Highly efficient and specific genome editing in silkworm using custom TALENs. *PLoS ONE* 7(9), e45035.

Ma, S., Liu, Y., Chang, J., Zhang, T., Wang, X. *et al.* (2017a) An integrated CRISPR *Bombyx mori* genome editing system with improved efficiency and expanded target sites. *Insect Biochemistry and Molecular Biology* 83, 13–20.

Ma, S., Xia, X., Li, Y., Sun, L., Liu, Y. *et al.* (2017b) Increasing the yield of middle silk gland expression system through transgenic knock-down of endogenous sericin-1. *Molecular Genetics and Genomics* 292(4), 823–831.

Minagawa, S., Nakaso, Y., Tomita, M., Igarashi, T., Miura, Y. *et al.* (2018) Novel recombinant feline interferon carrying N-glycans with reduced allergy risk produced by a transgenic silkworm system. *BMC Veterinary Research* 14(1), 260.

Minagawa, S., Sekiguchi, S., Nakaso, Y., Igarashi, T. and Tomita, M. (2020) Production of a correctly assembled fibrinogen using transgenic silkworms. *Transgenic Research* 29(3), 339–353.

Nakade, S., Tsubota, T., Sakane, Y., Kume, S., Sakamoto, N. *et al.* (2014) Microhomology-mediated end-joining-dependent integration of donor DNA in cells and animals using TALENs and CRISPR/Cas9. *Nature Communications* 5, 5560.

Ogawa, S., Tomita, M., Shimizu, K. and Yoshizato, K. (2007) Generation of a transgenic silkworm that secretes recombinant proteins in the sericin layer of cocoon: production of recombinant human serum albumin. *Journal of Biotechnology* 128(3), 531–544.

Okamoto, H., Ishikawa, E. and Suzuki, Y. (1982) Structural analysis of sericin genes. Homologies with fibroin gene in the 5' flanking nucleotide sequences. *Journal of Biological Chemistry* 257(24), 15192–15199.

Osanai-Futahashi, M., Ohde, T., Hirata, J., Uchino, K., Futahashi, R. *et al.* (2012a) A visible dominant marker for insect transgenesis. *Nature Communications* 3, 1295.

Osanai-Futahashi, M., Tatematsu, K.I., Yamamoto, K., Narukawa, J., Uchino, K. *et al.* (2012b) Identification of the *Bombyx* red egg gene reveals involvement of a novel transporter family gene in late steps of the insect ommochrome biosynthesis pathway. *Journal of Biological Chemistry* 287(21), 17706–17714.

Osanai-Futahashi, M., Tatematsu, K.I., Futahashi, R., Narukawa, J., Takasu, Y. *et al.* (2015) Positional cloning of a *Bombyx* pink-eyed white egg locus reveals the major role of cardinal in ommochrome synthesis. *Heredity (Edinburgh)* 116(2), 135–145.

Qian, W., Yang, Y., Li, Z., Wu, Y., He, X. *et al.* (2021) Enhanced Myc expression in silkworm silk gland promotes DNA replication and silk production. *Insects* 12(4), 361.

Quan, G.X., Kanda, T. and Tamura, T. (2002) Induction of the white egg 3 mutant phenotype by injection of the double-stranded RNA of the silkworm white gene. *Insect Molecular Biology* 11(3), 217–222.

Quan, G.-X., Kobayashi, I., Kojima, K., Uchino, K., Kanda, T. *et al.* (2007) Rescue of white egg 1 mutant by introduction of the wild-type *Bombyx* kynurenine 3-monooxygenase gene. *Insect Science* 14(2), 85–92.

Royer, C., Jalabert, A., Da Rocha, M., Grenier, A.M., Mauchamp, B. *et al.* (2005) Biosynthesis and cocoon-export of a recombinant globular protein in transgenic silkworms. *Transgenic Research* 14(4), 463–472.

Sajwan, S., Takasu, Y., Tamura, T., Uchino, K., Sezutsu, H. and Zurovec, M. (2013) Efficient disruption of endogenous *Bombyx* gene by TAL effector nucleases. *Insect Biochemistry and Molecular Biology* 43(1), 17–23.

Sakudoh, T., Sezutsu, H., Nakashima, T., Kobayashi, I., Fujimoto, H. *et al.* (2007) Carotenoid silk coloration is controlled by a carotenoid-binding protein, a product of the Yellow blood gene. *Proceedings of the National Academy of Sciences USA* 104(21), 8941–8946.

Sakudoh, T., Iizuka, T., Narukawa, J., Sezutsu, H., Kobayashi, I. *et al.* (2010) A CD36-related transmembrane protein is coordinated with an intracellular lipid-binding protein in selective carotenoid transport for cocoon coloration. *Journal of Biological Chemistry* 285(10), 7739–7751.

Sakudoh, T., Kuwazaki, S., Iizuka, T., Narukawa, J., Yamamoto, K. *et al.* (2013) CD36 homolog divergence is responsible for the selectivity of carotenoid species migration to the silk gland of the silkworm *Bombyx mori*. *Journal of Lipid Research* 54(2), 482–495.

Sakurai, T., Mitsuno, H., Haupt, S.S., Uchino, K., Yokohari, F. *et al.* (2011) A single sex pheromone receptor determines chemical response specificity of sexual behavior in the silkmoth *Bombyx mori*. *PLoS Genetics* 7(6), e1002115.

Sakurai, T., Mitsuno, H., Mikami, A., Uchino, K., Tabuchi, M. *et al.* (2015) Targeted disruption of a single sex pheromone receptor gene completely abolishes in vivo pheromone response in the silkmoth. *Science Reports* 5, 11001.

Saotome, T., Hayashi, H., Tanaka, R., Kinugasa, A., Uesugi, S. *et al.* (2015) Introduction of VEGF or RGD sequences improves revascularization properties of *Bombyx mori* silk fibroin produced by transgenic silkworm. *Journal of Materials Chemistry B* 3(35), 7109–7116.

Sato, M., Kojima, K., Sakuma, C., Murakami, M., Aratani, E. *et al.* (2012) Production of scFv-conjugated affinity silk powder by transgenic silkworm technology. *PLoS ONE* 7(4), e34632.

Sato, M., Kojima, K., Sakuma, C., Murakami, M., Tamada, Y. and Kitani. H. (2014) Production of scFv-conjugated affinity silk film and its application to a novel enzyme-linked immunosorbent assay. *Science Reports* 4, 4080.

Sato, M., Kitani, H. and Kojima, K. (2017) Development and validation of scFv-conjugated affinity silk protein for specific detection of carcinoembryonic antigen. *Science Reports* 7(1), 16077.

Saviane, A., Romoli, O., Bozzato, A., Freddi, G., Cappelletti, C. *et al.* (2018) Intrinsic antimicrobial properties of silk spun by genetically modified silkworm strains. *Transgenic Research* 27(1), 87–101.

Sezutsu, H., Sumitani, M., Kondo, M., Kobayashi, I., Takasu, Y. *et al.* (2018) [Construction of a platform for the development of pharmaceutical and medical applications using transgenic silkworms]. *Yakugaku Zasshi* 138(7), 863–874.

Shen, G., Wu, J., Wang, Y., Liu, H., Zhang, H. *et al.* (2018) The expression of ecdysteroid UDP-glucosyl-transferase enhances cocoon shell ratio by reducing ecdysteroid titre in last-instar larvae of silkworm, *Bombyx mori*. *Science Reports* 8(1), 17710.

Shimizu, K. (2018) Genetic engineered color silk: fabrication of a photonics material through a bioassisted technology. *Bioinspiration & Biomimetics* 13(4), 041003.

Shimomura, M., Minami, H., Suetsugu, Y., Ohyanagi, H., Satoh, C. *et al.* (2009) KAIKObase: an integrated silkworm genome database and data mining tool. *BMC Genomics* 10, 486.

Subbaiah, E.V., Royer, C., Kanginakudru, S., Satyavathi, V.V., Babu, A.S. *et al.* (2013) Engineering silkworms for resistance to baculovirus through multigene RNA interference. *Genetics* 193(1), 63–75.

Sun, Q., Jiang, L., Guo, H., Xia, F., Wang, B. *et al.* (2018) Increased antiviral capacity of transgenic silkworm via knockdown of multiple genes on *Bombyx mori* bidensovirus. *Developmental & Comparative Immunology* 87, 188–192.

Suzuki, M.G., Funaguma, S., Kanda, T., Tamura, T. and Shimada, T. (2003) Analysis of the biological functions of a doublesex homologue in *Bombyx mori*. *Development Genes and Evolution* 213(7), 345–354.

Suzuki, Y. and Brown, D.D. (1972) Isolation and identification of the messenger RNA for silk fibroin from *Bombyx mori*. *Journal of Molecular Biology* 63(3), 409–429.

Suzuki, Y., Gage, L.P. and Brown, D.D. (1972) The genes for silk fibroin in *Bombyx mori*. *Journal of Molecular Biology* 70(3):637–649.

Suzuki, Y., Tsuda, M., Takiya, S., Hirose, S., Suzuki, E. *et al.* (1986) Tissue-specific transcription enhancement of the fibroin gene characterized by cell-free systems. *Proceedings of the National Academy of Sciences USA* 83(24), 9522–9526.

Tada, M., Tatematsu, K., Ishii-Watabe, A., Harazono, A., Takakura, D. *et al.* (2015) Characterization of anti-CD20 monoclonal antibody produced by transgenic silkworms (*Bombyx mori*). *mAbs* 7(6), 1138–1150.

Takasu, Y., Yamada, H., Tamura, T., Sezutsu, H., Mita, K. and Tsubouchi, K. (2007) Identification and characterization of a novel sericin gene expressed in the anterior middle silk gland of the silkworm *Bombyx mori*. *Insect Biochemistry and Molecular Biology* 37(11), 1234–1240.

Takasu, Y., Kobayashi, I., Beumer, K., Uchino, K., Sezutsu, H. *et al.* (2010) Targeted mutagenesis in the silkworm *Bombyx mori* using zinc finger nuclease mRNA injection. *Insect Biochemistry and Molecular Biology* 40(10), 759–765.

Takasu, Y., Sajwan, S., Daimon, T., Osanai-Futahashi, M., Uchino, K. *et al.* (2013) Efficient TALEN construction for *Bombyx mori* gene targeting. *PLoS ONE* 8(9), e73458.

Takasu, Y., Tamura, T., Sajwan, S., Kobayashi, I. and Zurovec, M. (2014) The use of TALENs for nonhomologous end joining mutagenesis in silkworm and fruitfly. *Methods* 69(1), 46–57.

Takasu, Y., Kobayashi, I., Tamura, T., Uchino, K., Sezutsu, H. and Zurovec, M. (2016) Precise genome editing in the silkworm *Bombyx mori* using TALENs and ds- and ssDNA donors – a practical approach. *Insect Biochemistry and Molecular Biology* 78, 29–38.

Tamura, T., Kanda, T., Takiya, S., Okano, K. and Maekawa, H. (1990) Transient expression of chimeric CAT genes injected into early embryos of the domesticated silkworm *Bombyx mori*. *Japanese Journal of Genetics* 65(6), 401–410.

Tamura, T., Thibert, C., Royer, C., Kanda, T., Abraham, E. *et al.* (2000) Germline transformation of the silkworm *Bombyx mori* L. using a *piggyBac* transposon-derived vector. *Nature Biotechnology* 18(1), 81–84.

Tamura, T., Kuwabara, K., Uchino, K., Kobayashi, I. and Kanda, T. (2007) An improved DNA injection method for silkworm eggs drastically increases the efficiency of producing transgenic silkworms. *Journal of Insect Biotechnology and Sericology* 76, 155–159.

Tan, A., Tanaka, H., Tamura, T. and Shiotsuki, T. (2005) Precocious metamorphosis in transgenic silkworms overexpressing juvenile hormone esterase. *Proceedings of the National Academy of Sciences USA* 102(33), 11751–11756.

Tan, A., Fu, G., Jin, L., Guo, Q., Li, Z. *et al.* (2013) Transgene-based, female-specific lethality system for genetic sexing of the silkworm, *Bombyx mori*. *Proceedings of the National Academy of Sciences USA* 110(17), 6766–6770.

Tang, X., Liu, H., Shi, Z., Chen, Q., Kang, X. *et al.* (2020) Enhanced silk yield in transgenic silkworm (*Bombyx mori*) via ectopic expression of BmGT1-L in the posterior silk gland. *Insect Molecular Biology* 29(5), 452–465.

Tatematsu, K., Kobayashi, I., Uchino, K., Sezutsu, H., Iizuka, T. *et al.* (2009) Construction of a binary transgenic gene expression system for recombinant protein production in the middle silk gland of the silkworm *Bombyx mori*. *Transgenic Research* 19(3), 473–487.

Tatematsu, K., Yamamoto, K., Uchino, K., Narukawa, J., Iizuka, T. *et al.* (2011) Positional cloning of silkworm white egg 2 (w-2) locus shows functional conservation and diversification of ABC transporters for pigmentation in insects. *Genes to Cells* 16(4), 331–342.

Tatematsu, K., Sezutsu, H. and Tamura, T. (2012) Utilization of transgenic silkworms for recombinant protein production. *Journal of Biotechnology & Biomaterials* S9, 004. doi: 10.4172/2155-952X.S9-004

Tatematsu, K., Uchino, K., Sezutsu, H. and Tamura, T. (2014) Effect of ATG initiation codon context motifs on the efficiency of translation of mRNA derived from exogenous genes in the transgenic silkworm, *Bombyx mori*. *Springerplus* 3, 136.

Tatsuke, T., Lee, J.M., Kusakabe, T., Iiyama, K., Sezutsu, H. and Uchino, K. (2013) Tightly controlled tetracycline-inducible transcription system for explosive gene expression in cultured silkworm cells. *Archives of Insect Biochemistry and Physiology* 82(4), 173–182.

Teramoto, H. and Kojima, K. (2014) Production of *Bombyx mori* silk fibroin incorporated with unnatural amino acids. *Biomacromolecules* 15(7), 2682–2690.

Teramoto, H., Nakajima, K.I. and Kojima, K. (2016) Azide-incorporated clickable silk fibroin materials with the ability to photopattern. *ACS Biomaterials Science & Engineering* 2(2), 251–258.

Teramoto, H., Amano, Y., Iraha, F., Kojima, K., Ito, T. and Sakamoto, K. (2018) Genetic code expansion of the silkworm *Bombyx mori* to functionalize silk fiber. *ACS Synthetic Biology* 7(3), 801–806.

Teramoto, H., Iga, M., Tsuboi, H. and Nakajima, K. (2019) Characterization and scaled-up production of azido-functionalized silk fiber produced by transgenic silkworms with an expanded genetic code. *International Journal of Molecular Science* 20(3), 616.

Teramoto, H., Shirakawa, M. and Tamada, Y. (2020) Click decoration of *Bombyx mori* silk fibroin for cell adhesion control. *Molecules* 25(18).

Terenius, O., Papanicolaou, A., Garbutt, J.S., Eleftherianos, I., Huvenne, H. *et al.* (2010) RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design. *Journal of Insect Physiology* 57(2), 231–245.

Teule, F., Miao, Y.G., Sohn, B.H., Kim, Y.S., Hull, J.J. *et al.* (2012) Silkworms transformed with chimeric silkworm/spider silk genes composite silk fibers with improved mechanical properties. *Proceedings of the National Academy of Sciences USA* 109(3), 923–928.

Thomas, J.L., Da Rocha, M., Besse, A., Mauchamp, B. and Chavancy, G. (2002) 3xP3-EGFP marker facilitates screening for transgenic silkworm *Bombyx mori* L. from the embryonic stage onwards. *Insect Biochemistry and Molecular Biology* 32(3), 247–253.

Tomihara, K., Satta, K., Matsuzaki, S., Yoshitake, K., Yamamoto, K. *et al.* (2021) Mutations in a β group of solute carrier gene are responsible for egg and eye coloration of the brown egg 4 (b-4) mutant in the silkworm, *Bombyx mori*. *Insect Biochemistry and Molecular Biology* 137, 103624.

Tomita, M. (2010) Transgenic silkworms that weave recombinant proteins into silk cocoons. *Biotechnology Letters* 33(4), 645–654.

Tomita, M. (2018) [Development of large-scale silkworm-rearing technologies for the GMP production of biologics]. *Yakugaku Zasshi* 138(7), 875–884.

Tomita, M., Munetsuna, H., Sato, T., Adachi, T., Hino, R. *et al.* (2003) Transgenic silkworms produce recombinant human type III procollagen in cocoons. *Nature Biotechnology* 21(1), 52–56.

Tomita, M., Hino, R., Ogawa, S., Iizuka, M., Adachi, T. *et al.* (2007) A germline transgenic silkworm that secretes recombinant proteins in the sericin layer of cocoon. *Transgenic Research* 16(4), 449–465.

Tomita, S., Kanda, T., Imanishi, S. and Tamura, T. (1999) Yeast FLP recombinase-mediated excision in cultured cells and embryos of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *Applied Entomology and Zoology* 34, 371–377.

Tsubota, T. and Sezutsu, H. (2017) Genome editing of silkworms. In: Hatada, I. (ed.) *Genome Editing in Animals*. Series: Methods in Molecular Biology, Vol. 1630. Humana Press, New York, pp. 205–2118.

Tsubota, T., Uchino, K., Kamimura, M., Ishikawa, M., Hamamoto, H. *et al.* (2013) Establishment of transgenic silkworms expressing GAL4 specifically in the haemocyte oenocytoid cells. *Insect Molecular Biology* 23(2), 165–174.

Tsubota, T., Uchino, K., Suzuki, T.K., Tanaka, H., Kayukawa, T. *et al.* (2014) Identification of a novel strong and ubiquitous promoter/enhancer in the silkworm *Bombyx mori*. *G3: Genes, Genomes, Genetics* 4(7), 1347–1357.

Uchino, K., Imamura, M., Shimizu, K., Kanda, T. and Tamura, T. (2007) Germ line transformation of the silkworm, *Bombyx mori*, using the transposable element Minos. *Molecular Genetics and Genomics* 277(3), 213–220.

Uchino, K., Sezutsu, H., Imamura, M., Kobayashi, I., Tatematsu, K. *et al.* (2008) Construction of a *piggyBac*-based enhancer trap system for the analysis of gene function in silkworm *Bombyx mori*. *Insect Biochemistry and Molecular Biology* 38(12), 1165–1173.

Wang, F., Xu, H., Wang, Y., Wang, R., Yuan, L. *et al.* (2014b) Advanced silk material spun by a transgenic silkworm promotes cell proliferation for biomedical application. *Acta Biomaterialia* 10(12), 4947–4955.

Wang, F., Ji, Y.T., Tian, C., Wang, Y.C., Xu, S. *et al.* (2021) An inducible constitutive expression system in *Bombyx mori* mediated by phiC31 integrase. *Insect Science* 28(5), 1277–1289.

Wang, L., Kiuchi, T., Fujii, T., Daimon, T., Li, M. *et al.* (2013b) Mutation of a novel ABC transporter gene is responsible for the failure to incorporate uric acid in the epidermis of ok mutants of the silkworm, Bombyx mori. *Insect Biochemistry and Molecular Biology* 43(7), 562–571.

Wang, Y., Li, Z., Xu, J., Zeng, B., Ling, L. *et al.* (2013a) The CRISPR/Cas system mediates efficient genome engineering in Bombyx mori. *Cell Research* 23(12), 1414–1416.

Wang, Y., Tan, A., Xu, J., Li, Z., Zeng, B. *et al.* (2014a). Site-specific, TALENs-mediated transformation of Bombyx mori. *Insect Biochemistry and Molecular Biology* 55, 26–30.

Wang, Y., Wang, Z., Guo, H., Huang, J., Li, X. *et al.* (2019b) Potential of transferring transgenic DNA from silkworm to chicken. *International Journal of Biological Macromolecules* 142, 311–319.

Wang, Z., Guo, P., Wang, Q., Zhang, Y., Xu, H. and Zhao P. (2019a) Overexpression of Gloverin2 in the Bombyx mori silk gland enhances cocoon/silk antimicrobial activity. *Development of Comparative Immunology* 98, 6–12.

Wei, W., Xin, H., Roy, B., Dai, J., Miao, Y. and Gao, G. (2014) Heritable genome editing with CRISPR/Cas9 in the silkworm, *Bombyx mori*. *PLoS ONE* 9(7), e101210.

Wen, H., Lan, X., Zhang, Y., Zhao, T., Wang, Y. *et al.* (2010) Transgenic silkworms (*Bombyx mori*) produce recombinant spider dragline silk in cocoons. *Molecular Biology Reports* 37(4), 1815–1821.

Woltje, M., Bobel, M., Bienert, M., Neuss, S., Aibibu, D. and Cherif, C. (2018) Functionalized silk fibers from transgenic silkworms for wound healing applications: surface presentation of bioactive epidermal growth factor. *Journal of Biomedical Materials Research A* 106(10), 2643–2652.

Wu, M., Huang, S., Ye, X., Ruan, J., Zhao, S. *et al.* (2021) Human epidermal growth factor-functionalized cocoon silk with improved cell proliferation activity for the fabrication of wound dressings. *Journal of Biomaterials Applications* 36(4), 722–730.

Xu, H. (2014) The advances and perspectives of recombinant protein production in the silk gland of silkworm *Bombyx mori*. *Transgenic Research* 23(5), 697–706.

Xu, H. and O'Brochta, D.A. (2015) Advanced technologies for genetically manipulating the silkworm *Bombyx mori*, a model Lepidopteran insect. *Proceedings of the Royal Society B: Biological Sciences* 282(1810), 20150487.

Xu, J., Bi, H., Chen, R., Aslam, A.F., Li, Z. *et al.* (2014a) Transgenic characterization of two testis-specific promoters in the silkworm, *Bombyx mori. Insect Molecular Biology* 24(2), 183–190.

Xu, J., Wang, Y.Q., Li, Z.Q., Ling, L., Zeng, B.S. *et al.* (2014b) Functional characterization of the vitellogenin promoter in the silkworm, *Bombyx mori. Insect Molecular Biology* 23(5), 550–557.

Xu, J., Wang, Y., Li, Z., Ling, L., Zeng, B. *et al.* (2014c) Transcription activator-like effector nuclease (TALEN)-mediated female-specific sterility in the silkworm, *Bombyx mori. Insect Molecular Biology* 23(6), 800–807.

Xu, J., Dong, Q., Yu, Y., Niu, B., Ji, D. *et al.* (2018) Mass spider silk production through targeted gene replacement in *Bombyx mori*. *Proceedings of the National Academy of Sciences USA* 115(35), 8757–8762.

Xu, J., Chen, R.-M., Chen, S.-Q., Chen, K., Tang, L.-M. *et al.* (2019a) Identification of a germline-expression promoter for genome editing in *Bombyx mori*. *Insect Science* 26(6), 991–999.

Xu, J., Luo, X., Fang, G., Zhan, S., Wu, J. *et al.* (2020) Transgenic expression of antimicrobial peptides from black soldier fly enhance resistance against entomopathogenic bacteria in the silkworm, Bombyx mori. *Insect Biochemistry and Molecular Biology* 127, 103487.

Xu, S., Wang, F., Wang, Y., Wang, R., Hou, K. *et al.* (2019b) A silkworm based silk gland bioreactor for high-efficiency production of recombinant human lactoferrin with antibacterial and anti-inflammatory activities. *Journal of Biological Engineering* 13, 61.

Yamada, Y., Matsuyama, T., Quan, G.X., Kanda, T., Tamura, T. *et al.* (2002) Use of an N-terminal half truncated IE1 as an antagonist of IE1, an essential regulatory protein in baculovirus. *Virus Research* 90 (1-2), 253–261.

Yamada, K., Masuda, K., Ida, S., Tada, H., Bando, M. *et al.* (2021) In vitro assessment of antitumor immune responses using tumor antigen proteins produced by transgenic silkworms. *Journal of Materials Science: Materials in Medicine* 32(6), 58.

Yamagata, T., Sakurai, T., Uchino, K., Sezutsu, H., Tamura, T. and Kanzaki, R. (2008) GFP labeling of neurosecretory cells with the GAL4/UAS system in the silkmoth brain enables selective intracellular staining of neurons. *Zoological Science* 25(5), 509–516.

Yanagisawa, S., Zhu, Z., Kobayashi, I., Uchino, K., Tamada, Y. *et al.* (2007) Improving cell-adhesive properties of recombinant *Bombyx mori* silk by incorporation of collagen or fibronectin derived peptides produced by transgenic silkworms. *Biomacromolecules* 8(11), 3487–3492.

Yang, J.G., Liu, T.H., Dong, X.L., Wu, Y.F., Zhang, Q. *et al.* (2017) In vivo RNA interference of BmNHR96 enhances the resistance of transgenic silkworm to BmNPV. *Biochemical and Biophysical Research Communications* 493(1), 332–339.

Yin, Y., Cao, G., Xue, R. and Gong, C. (2014) Construction of transformed, cultured silkworm cells and transgenic silkworm using the site-specific integrase system from phage phiC31. *Molecular Biology Reports* 41(10), 6449–6456.

Yoda, S., Yamaguchi, J., Mita, K., Yamamoto, K., Banno, Y. *et al.* (2014) The transcription factor Apontic-like controls diverse colouration pattern in caterpillars. *Nature Communications* 5, 4936.

Yonemura, N., Tamura, T., Uchino, K., Kobayashi, I., Tatematsu, K. *et al.* (2012) PhiC31 integrase-mediated cassette exchange in silkworm embryos. *Molecular Genetics and Genomics* 287(9), 731–739.

Yonemura, N., Tamura, T., Uchino, K., Kobayashi, I., Tatematsu, K. *et al.* (2013) PhiC31-integrase-mediated, site-specific integration of transgenes in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *Applied Entomology and Zoology* 48, 265–273.

You, L., Zhang, F., Huang, S., Merchant, A., Zhou, X. and Li, Z. (2020) Over-expression of RNA interference (RNAi) core machinery improves susceptibility to RNAi in silkworm larvae. *Insect Molecular Biology* 29(4), 353–362.

You, Z., Ye, X., Ye, L., Qian, Q., Wu, M. *et al.* (2018) Extraordinary mechanical properties of composite silk through hereditable transgenic silkworm expressing recombinant major ampullate spidroin. *Scientific Reports* 8(1), 15956.

Yuasa, M., Kiuchi, T., Banno, Y., Katsuma, S. and Shimada, T. (2015) Identification of the silkworm quail gene reveals a crucial role of a receptor guanylyl cyclase in larval pigmentation. *Insect Biochemistry and Molecular Biology* 68, 33–40.

Yukuhiro, K., Sezutsu, H., Tamura, T., Kosegawa, E., Iwata, K. *et al.* (2013) Little gene flow between domestic silkmoth *Bombyx mori* and its wild relative *Bombyx mandarina* in Japan, and possible artificial selection on the CAD gene of B. mori. *Genes & Genetic Systems* 87(5), 331–340.

Zabelina, V., Klymenko, V., Tamura, T., Doroshenko, K., Liang, H. *et al.* (2015a) Genome engineering and parthenocloning in the silkworm, *Bombyx mori. Journal of Bioscience* 40(3), 645–655.

Zabelina, V., Uchino, K., Mochida, Y., Yonemura, N., Klymenko, V. *et al.* (2015b) Construction and long term preservation of clonal transgenic silkworms using a parthenogenetic strain. *Journal of Insect Physiology* 81, 28–35.

Zabelina, V., Takasu, Y., Sehadova, H., Yonemura, N., Nakajima, K. *et al.* (2021) Mutation in *Bombyx mori* fibrohexamerin (P25) gene causes reorganization of rough endoplasmic reticulum in posterior silk gland cells and alters morphology of fibroin secretory globules in the silk gland lumen. *Insect Biochemistry and Molecular Biology* 135, 103607.

Zhang, H., Kiuchi, T., Wang, L., Kawamoto, M., Suzuki, Y. *et al.* (2017a) Bm-muted, orthologous to mouse muted and encoding a subunit of the BLOC-1 complex, is responsible for the otm translucent mutation of the silkworm *Bombyx mori*. *Gene* 629, 92–100.

Zhang, P., Wang, J., Lu, Y., Hu, Y., Xue, R. *et al.* (2013) Resistance of transgenic silkworm to BmNPV could be improved by silencing ie-1 and lef-1 genes. *Gene Therapy* 21(1), 81–88.

Zhang, P., Liu, S., Song, H.S., Zhang, G., Jia, Q. and Li, S. (2017b) Yorkie(CA) overexpression in the posterior silk gland improves silk yield in *Bombyx mori*. *Journal of Insect Physiology* 100, 93–99.

Zhang, T., Liu, R., Luo, Q., Qu, D., Chen, T. *et al.* (2019a) Expression and characterization of recombinant human VEGF165 in the middle silk gland of transgenic silkworms. *Transgenic Research* 28(5-6), 601–609.

Zhang, X., Xia, L., Day, B.A., Harris, T.I., Oliveira, P. *et al.* (2019b) CRISPR/Cas9 initiated transgenic silkworms as a natural spinner of spider silk. *Biomacromolecules* 20(6), 2252–2264.

Zhang, Y., Hu, J., Miao, Y., Zhao, A., Zhao, T.*eet al*. (2008) Expression of EGFP-spider dragline silk fusion protein in BmN cells and larvae of silkworm showed the solubility is primary limit for dragline proteins yield. *Molecular Biology Reports* 35(3), 329–335.

Zhang, Z., Niu, B., Ji, D., Li, M., Li, K. *et al.* (2018) Silkworm genetic sexing through W chromosome-linked, targeted gene integration. *Proceedings of the National Academy of Sciences USA* 115(35), 8752–8756.

Zhao, P., Xia, F., Jiang, L., Guo, H., Xu, G. *et al.* (2018) Enhanced antiviral immunity against *Bombyx mori* cytoplasmic polyhedrosis virus via overexpression of peptidoglycan recognition protein S2 in transgenic silkworms. *Developmental Comparative Immunology* 87, 84–89.

Zhao, S., Ye, X., Wu, M., Ruan, J., Wang, X. *et al.* (2021) Recombinant silk proteins with additional polyalanine have excellent mechanical properties. *International Journal of Molecular Sciences* 22(4), 1513.

Zhou, C.Z., Confalonieri, F., Medina, N., Zivanovic, Y., Esnault, C. *et al.* (2000) Fine organization of *Bombyx mori* fibroin heavy chain gene. *Nucleic Acids Research* 28(12), 2413–2419.

21 Tephritid Fruit Fly Transgenesis and Applications

Alfred M. Handler[1]* and Marc F. Schetelig[2]

[1]*USDA/ARS, Center for Medical, Agricultural and Veterinary Entomology, Gainesville, Florida;* [2]*Justus-Liebig-University Gießen, Department of Insect Biotechnology in Plant Protection, Gießen, Germany*

21.1 Introduction

Tephritid fruit flies are among the most serious agricultural pests in the world, owing in large part to those species having broad host ranges including hundreds of fruits and vegetables. They are the largest group of insects subject to population control by biologically based systems, most notably the sterile insect technique (SIT) (Mitchell and Saul, 1990) (see Scott *et al.*, Chapter 17, this volume, for a brief overview of the SIT). Given the needs for improved SIT, including visible markers for field detection, sexing systems for male-only strains and male sterilization, the possibility of achieving these improvements using genetically modified strains has been a long-standing goal (Robinson and Franz, 2000). Thus, it is not surprising that the first report of a transposon-mediated germline transformation of a non-drosophilid insect species was for the tephritid Mediterranean fruit fly, *Ceratitis capitata* (medfly) (Loukeris *et al.*, 1995a).

The success of this transformation, after many attempts by several laboratories using the *Drosophila P* and *hobo* vector systems, was due largely to the use of the newly discovered and widely active *Tc*-related *Minos* element (see O'Brochta, Chapter 1, this volume) (Franz and Savakis, 1991). Of equal importance for transformation of this species were the availability of a *white eye* (*we*) mutant host strain (McCombs and Saul, 1992; Rossier and Rosenthal, 1992) and the cloning of the medfly *we*+ allele that could be used as mutant rescue marker for transformant selection (Zwiebel *et al.*, 1995). This same marker system was then successfully used to transform medfly with the *piggyBac* (Handler *et al.*, 1998) and *Hermes* (Michel *et al.*, 2001) transposon vector systems. In subsequent years, at least five additional tephritid species have been transformed with *piggyBac* or *Minos*, though typically using the more widely applicable fluorescent protein (FP) markers (Horn *et al.*, 2002). Together, germline transformation of tephritid species represents the largest number of species from a single family, which has been due in large part to the economic interest in these species (Handler, 2002a) and the relative ease of using methods and reagents for transgenesis that have been developed for *Drosophila* (Handler, 2000).

For the purpose of this chapter, we focus on the first transformation experiments

---

*Corresponding author, email: ahandler@ufl.edu

for a particular vector system for tephritid species in which it has been tested. Additional experiments are discussed as they relate to new marker systems and vectors developed for particular applications and, in particular, CRISPR/Cas9 homology-directed repair (HDR) gene insertions and substitutions. These include vector stabilization systems, site-specific genomic targeting and, finally, the development of tephritid transgenic strains for control applications.

21.2 Transformation with the *Minos* Vector System

Minos was originally discovered in *Drosophila hydei* as part of a ribosomal RNA transcriptional unit (Franz and Savakis, 1991) and analysis of several *Minos* elements showed that the functional element had a transposase encoded by two exons surrounded by 254 bp inverted terminal repeats (Franz *et al*., 1994). *Minos* is a member of the *Tc* family, having > 40% coding sequence identity with *Tc*1, and causes a TA duplication of its insertion site. The ability of *Minos* to function as a transformation vector was first tested in *Drosophila melanogaster* using a *white*[+] marked vector and an *hsp70*-regulated helper (Loukeris *et al*., 1995a). While transformation frequencies in the range of about 5% per fertile G_0 were relatively low, transformants were consistently produced in several experiments. The relationship of *Minos* to *Tc*1 transposon originally discovered in a nematode (and subsequently found to be part of a broad-ranging transposon family including *mariner* elements) suggested that it might function, unlike *P*, in non-drosophilid insects.

21.2.1 *Minos* transformation of the Mediterranean fruit fly, *Ceratitis capitata*

The availability of a cDNA clone for the *white eye*[+] gene from the medfly (also referred to as *white*[+], which is its orthologue in *Drosophila*) (Zwiebel *et al*., 1995) made it possible to

test *Minos* vector function in a medfly *white eye* host strain (McCombs and Saul, 1992) using *Drosophila* protocols (Loukeris *et al*., 1995b). Similar to the *Drosophila* transformation experiments (Rubin and Spradling, 1982), a vector carrying an *hsp70*-regulated *we*[+] cDNA marker was co-injected with the *hsp70-Minos* transposase helper. Several transformant lines were generated at an overall frequency of < 5%, though this is an estimate (due to group matings of the G_1 flies). Nevertheless, transformations were verified by Southern blot hybridization, indicating that bona fide transposon-mediated transformants had been generated. Thus, the *Minos* transposable element was the first vector system to successfully transform the germline of a non-drosophilid species, the tephritid *C. capitata* (Loukeris *et al*., 1995b).

21.2.2 *Minos* transformation of the olive fruit fly, *Bactrocera oleae*

The olive fruit fly (olive fly) *Bactrocera oleae* is a highly destructive pest of olives throughout the world and has recently been introduced in the west coast of the USA. While SIT has been highly successful in controlling populations of several tephritid species, its use in olive fly has presented some unique difficulties that might be overcome by transgenic approaches. In particular would be genetic sterilization without the fitness and mating competition costs of radiation and sexing that would allow male-only releases. Towards that end, the first germline transformation of *B. oleae* was achieved with a uniquely marked *Minos* vector (Koukidou *et al*., 2006). For this vector, pMiBO14/GtTA2, a marker was constructed having a tetracycline-suppressible cassette with both enhanced green fluorescent protein (*EGFP*) and the Tet-transactivator (*tTA*) linked in a bidirectional manner to a 14-mer tet response element, thereby creating a self-promoting and highly expressing EGFP marking system. Also unique to this transformation system was the direct use of *Minos* transposase mRNA to catalyse transposition, rather than

a helper plasmid, which was co-injected into embryos with the vector. From over 3800 injected embryos, 325 G_0 adults survived that were backcrossed to wild-type (non-transformed) males or females in cages. A total of 67 EGFP-expressing G_1 flies were screened, from which 13 separate lines were created and analysed by Southern hybridization and inverse PCR, which verified *Minos*-mediated transformations. While a precise transformation frequency could not be derived, due to group matings, differing patterns of fluorescence suggested that several independent lines were created, supporting the assumption that transformation was relatively robust. While the strength of this system may have resulted from either the self-promoting marker or use of transposase mRNA as helper, or both, these potential improvements have yet to be tested in other tephritid species.

21.3   Transformation with the *piggyBac* Vector System

The *piggyBac* IFP2 element was one of the few transposons discovered by virtue of its ability to transpose from a lepidopteran cell-line genome into an infectious baculovirus (Fraser *et al.*, 1983; Cary *et al.*, 1989). Unlike most other transposons that were discovered initially as a non-mobile variant of an autonomous functional element, the original IFP2 element was not only functional, but was also able to transpose in a prokaryotic system. This indicated that IFP2 itself might be functional as a transformation vector and that it might retain function in a wide variety of organisms. Both possibilities were supported by a series of cell line and organismal transient mobility assays with the element (Fraser *et al.*, 1995). This led to the first transformation with a *piggyBac* vector system in the medfly and it has since become the most widely used vector for tephritid species, as well as insects in five orders (Handler, 2002b; Handler and O'Brochta, 2012) (Table 21.1) (see also O'Brochta, Chapter 1, this volume).

21.3.1   *piggyBac* transformation of the Mediterranean fruit fly, *Ceratitis capitata*

The *piggyBac* IFP2 transposon was first tested as a transformation vector in the medfly. Similar to the *Minos* transformation, a vector, pB[*Ccw*+], was created by inserting the *hsp70*-regulated medfly *white eye*+ gene cDNA into the unique *Hpa*I site in the *piggyBac* transposase-coding region, thereby eliminating transposase production from the vector (Handler *et al.*, 1998). The first *piggyBac* transposase helper, pBΔSacI, was regulated by its own promoter, and made non-mobile by a *Sac*I deletion of the 5′ terminal sequence of the IFP2 element within the p3E1.2 plasmid. As is typical for *Drosophila P* vector transformations (Rubin and Spradling, 1982), medfly *white eye* host strain embryos were injected with 500:150 μg/ml and 500:300 μg/ml mixtures of the vector and helper plasmids, respectively. From the two experiments, six transformant lines were generated that were initially identified by varying levels of eye pigmentation, presumably due to genomic position effect suppression. Transformation therefore occurred at an approximate frequency of 3.5% per fertile G_0 individual. These were verified as *piggyBac*-mediated events by Southern hybridization that allowed a determination of one or two vector insertions in each G_1 sub-line. Insertion site sequencing by inverse PCR of the vector termini/genomic junctions confirmed duplication of the TTAA insertion site sequences characteristic of *piggyBac* transposition.

An improved *piggyBac* helper plasmid, phsp-pBac, was subsequently created by inserting the *D. melanogaster hsp70* gene promoter upstream to the *piggyBac* promoter in the *Sac*I deletion within pBΔSac. The two helpers were first compared in *D. melanogaster*, where phsp-pBac yielded at least an eightfold higher frequency of transformation (Handler and Harrell, 1999). While actual transformation frequencies can vary widely, phsp-pBac has proven to be highly efficient in generating transformants in medfly and is now routinely used for most dipteran transformations, including most of

Table 21.1. Transposon-mediated germline transformations of tephritid fruit fly species. Transformations listed represent the first use of a transposon vector with a specific marker in the species.

Tephritid species	Transposon vector	Marker	Reference
Ceratitis capitata	*Hermes*	*Cc-white*[+]	Michel *et al.*, 2001
	Minos	*Cc-white*[+]	Loukeris *et al.*, 1995b
	piggyBac	*Cc-white*[+]	Handler *et al.*, 1998
	piggyBac	*ubi-p63E-DsRed2 ie1-DsRed2*	Gong *et al.*, 2005
	piggyBac	*hr5-IE1-CopGreen/PhiYFP/J-Red*	Dafa'alla *et al.*, 2006
	piggyBac	*Ccβ2t-DsRedEx, Ccβ2t-tGFP*	Scolari *et al.*, 2008
	piggyBac	*PUb-DsRed1*	Schetelig *et al.*, 2009a
	piggyBac	*PUb-nls-EGFP*	A. Handler and R. Krasteva, unpublished
Anastrepha ludens	*piggyBac*	*ubiquitin-CopGreen/ PhiYFP/J-Red*	Condon *et al.*, 2007a
		PUb-nls-EGFP/PUb-DsRed.T3	Meza *et al.*, 2011
		Asβ2tub-DsRed.T3	Meza *et al.,* 2014b
Anastrepha suspensa	*piggyBac*	*PUb-nls-EGFP*	Handler and Harrell, 2001b
	piggyBac	*PUb-DsRed1*	Handler and Harrell, 2001a
	piggyBac	*Asβ2tub-DsRed.T3*	Zimowska *et al.*, 2009
	piggyBac	*Pub-nls-EGFP/3xP3-FRT-AmCyan*	Schetelig and Handler, 2013a
	hopper	*PUbDsRed.T3*	Handler and Schetelig, 2020
Bactrocera dorsalis	*piggyBac*	*Cc-white*[+]	Handler and McCombs, 2000
	piggyBac	*PUb-nls-EGFP, Cc-white*[+]	A. Handler and S. McCombs, unpublished
Bactrocera oleae	*Minos*	*tTA/EGFP*	Koukidou *et al.*, 2006
	piggyBac	*hr5-IE1-DsRed2*	Ant *et al.*, 2012
	piggyBac	*PUb-nls-EGFP/PUb-DsRed.T3*	Genc *et al.,* 2016
Bactrocera tryoni	*piggyBac*	*PUb-nls-EGFP/PUb-DsRed*	Raphael *et al.*, 2011

the other tephritid *piggyBac* transformations discussed in this report. Use of this promoter was initially accompanied by a 45–60 min heat shock at 37°C within 1 day of injection, but heat shock has typically been eliminated, resulting in similar rates of transformation with better G_0 embryo survival.

21.3.2 *piggyBac* transformation of the oriental fruit fly, *Bactrocera dorsalis*

A high degree of homology between the *white eye* genes in *B. dorsalis* and *C. capitata* was inferred by the similarity of the genes in medfly and *Drosophila* (Zwiebel *et al.*, 1995). Thus, it was presumed that the medfly *we*[+] gene could be used as a selectable marker for *piggyBac* transformation of a *white eye* mutant oriental fruit fly (*Bactrocera dorsalis*)

strain selected from a wild-type Hawaiian strain (McCombs and Saul, 1992). For this transformation, the pB[*Ccw*[+]] vector was co-injected with the phsp-pBac helper, yielding three putative transformant lines from 102 fertile G_0s, resulting in a similar transformation frequency as that achieved in medfly (Handler and McCombs, 2000). Notably, one of the G_1 lines with pigmented eyes was extremely pale, and almost indistinguishable from the mutant host *white eye* phenotype, but this line and the two others were verified as transformants by Southern hybridization. The hybridization analysis with a *piggyBac* probe, however, as well as hybridization to a vector-specific fragment, showed additional hybridization patterns in the transformant lines that were also present in non-transformed *white eye* and wild-type control samples. This suggested that *piggyBac* or *piggyBac*-like sequences exist in

B. dorsalis, an observation reaffirmed by hybridization and PCR analysis of several other *B. dorsalis* mutant and wild-type strains (Handler and McCombs, 2000). Indeed, nearly identical, though apparently non-functional, *piggyBac*-like elements were shown to exist in *B. dorsalis sensu stricto*, as well as several other species within the *B. dorsalis* species complex (Bonizzoni *et al.*, 2007; Handler *et al.*, 2008). Given the potential ambiguity of identifying *B. dorsalis* transformants with a *piggyBac* probe, the transformants were also verified by hybridization with a *white eye* probe, showing one or two integrations in the G_1 sub-lines. However, the presence of non-vector *piggyBac* sequences precluded straightforward insertion site sequencing by inverse PCR.

In a separate experiment, a *piggyBac* vector marked with medfly we^+ and *D. melanogaster polyubiquitin*-regulated *EGFP* was introduced into the *B. dorsalis white eye* strain, resulting in a single transformant line from 17 G_0 matings (A.M. Handler and S.D. McCombs, unpublished). As in *Drosophila*, the transformant was selected solely by GFP expression, having undetectable eye pigmentation. This reaffirms the notion that the *polyubiquitin-EGFP* marker, and presumably other fluorescent-protein markers, is significantly more reliable than w^+ or we^+ (see sections 21.3.3 and 21.6.1).

The discovery of *piggyBac*-like elements in *Bactrocera* was the first indication that the element exists outside the Lepidoptera. While this is not unexpected, given its autonomous function in dipterans, the presence of closely related *piggyBac* elements in dipterans is clearly discontinuous. This was indicated by the lack of *piggyBac* DNA hybridization to *Drosophila* and other tephritid species, including medfly and the melon fly *Zeugodacus cucurbitae*. A more complete assessment of *piggyBac* presence and function in insect (and non-insect) species is therefore very important in terms of determining potential vector stability in specific hosts. Nevertheless, *piggyBac* transformation did occur relatively efficiently in a species containing non-functional elements, in contrast to *Drosophila P* strains, where *P*-elements repress their own mobility (Engels, 2007).

21.3.3 *piggyBac* transformation of the Caribbean fruit fly, *Anastrepha suspensa*

To extend *piggyBac* gene transfer to other dipteran species, especially to those not having mutations for visible marking, a dominant-acting fluorescent protein marker system was tested in the Caribbean fruit fly (caribfly) *Anastrepha suspensa* (Handler and Harrell, 2001b). Previous to testing in the caribfly, for which no other marking system existed, a dual marked *piggyBac* vector, pB[*PUb-nls-EGFP, Dmw*⁺], was created with *D. melanogaster polyubiquitin* (*PUb*)-regulated *EGFP* and w^+ for testing in *D. melanogaster* (Handler and Harrell, 1999) (see section 21.6.1 for details on the marker construct). With this vector the EGFP marker could be tested using w^+ as a control, with the expectation that transformants identified by eye pigmentation would also express EGFP. Indeed, not only were all w^+ transformants also marked by whole-body expression of EGFP, but in addition the EGFP marker was easily detected in transformant larvae and, unexpectedly, a significant number of EGFP-expressing G_1 individuals did not express easily identifiable eye pigmentation in adults. Thus, the *PUb-nls-EGFP* marker proved to be a significant improvement over the w^+ marker in *Drosophila* in terms of earlier detection and sensitivity to position-effect suppression.

Successful transformant marking with *PUb-nls-EGFP* in *Drosophila* did not, however, ensure the same result for a tephritid species, especially since EGFP was regulated by a *Drosophila* promoter (Lee *et al.*, 1988; Davis *et al.*, 1995). To test this possibility, a *piggyBac* vector marked solely with *PUb-nls-EGFP* was co-injected with the phsp-pBac helper into caribfly embryos. From 60 group inter-matings of 561 surviving G_0 adults, four of the G_0 groups yielded 57 G_1 offspring, exhibiting green fluorescence at all stages of development. Genomic *piggyBac*-mediated vector integrations were verified by Southern hybridization, revealing at least six independent transformant lines, for which two were also verified by insertion-site sequencing. Notably, one transformant line contained four independent integrations, the most detected in any tephritid transformation.

The successful use of the *PUb-nls-EGFP* marker for the first time in a non-drosophilid was especially important in terms of its potential use in many other species not amenable to mutant-rescue marking, and the advantages of early detection. However, it became apparent that GFP and other fluorescent proteins could also act as important marker systems to monitor released males, and especially transgenics, used in SIT. This possibility was first tested in caribfly transformants by determining the length of time GFP fluorescence could be unambiguously identified in dead adults under simulated dry-trap conditions, which was between 2 to 3 weeks (Handler and Harrell, 2001b). This was studied in more detail in caribflies transformed with DsRed-marked vectors (see section 21.6.1). Given the potential use of fluorescent protein marking systems, and the need to identify transgenic lines having the strongest expression, a spectrofluorometric method to quantitatively determine relative fluorescence of transformant lines was developed (Handler and Harrell, 2001b). This method simply entailed homogenizing fluorescent flies in phosphate buffer and determining protein concentration and fluorescence in supernatant aliquots after centrifugation. By comparing spectrofluorometric readings to a standard curve, fluorescence relative to protein concentration could be quantitatively deduced for each transgenic line. For the caribfly transformants, relative spectrofluorometric fluorescence levels were generally consistent with epifluorescence visual observations and with transgene copy number.

21.3.4 *piggyBac* transformation of the Mexican fruit fly, *Anstrepha ludens*

The Mexican fruit fly (mexfly) *Anastrepha ludens* has been transformed using *piggyBac*-based vector systems in two studies from different laboratories. Coincidentally, both studies tested the use of new types of transposon vectors that had been validated previously in *Drosophila* or medfly, in which one or both inverted terminal repeat (ITR) sequences could

be deleted subsequent to or during vector transposition, respectively. Loss of vector ITRs results in immobilization of remaining vector sequences, thereby providing stability to genomic integrations, and these systems and their use are discussed in more detail below (see section 21.6).

The first mexfly transformation used two vectors having similar structure and marked with three fluorescent protein markers, CopGreen, PhiYFP and J-Red (Condon *et al.*, 2007a). Both CopGreen and J-Red were bounded by *piggyBac* 5′ and 3′ ITR sequences, making them independent vectors. A functional *piggyBac* transposase gene and the PhiYFP marker were inserted between the marker cassettes, making this an autonomous vector not requiring an exogenous transposase helper. Based on placement of the ITRs, four possible vector insertions were possible. The vectors were injected into 937 embryos in total, with 170 surviving G_0 adults pool-mated, resulting in 122 putative G_1 transformants based on screening for all three markers. While pool-matings precluded an accurate determination of transformation frequency, a minimum 4.3% frequency was deduced. Molecular analysis of transformants was limited to PCR of the internal fluorescent protein genes and thus *piggyBac*-mediated events could not be verified.

The second *piggyBac* transformation of mexfly used two different vectors as well, but one was a dual-marked stabilization vector and the other used *PUb-nls-EGFP* as a transformation marker with another marker having *DsRed* regulated by the *β2-tubulin* promoter for spermatocyte-specific expression (Meza *et al.*, 2011) (see sections 21.6.2 and 21.7 for details on vectors for sperm-marking and stabilization). The stabilization vector, pB[L1-*EGFP*-L2-*DsRed*-R1], has the *PUb-nls-EGFP* marker inserted in between a tandem duplication of the *piggyBac* 5′ ITR (L1 and L2), and *PUb-DsRed* inserted in between the internal 5′ ITR (L2) and the external 3′ ITR (R1). This vector was used with the phsp-pBac helper to generate 138 G_0 adults that were mated in 20 small groups (3–5 G_0s outcrossed to wild-type), resulting in putative G_1 transformants from 15 of the independent groups at an estimated frequency of approximately 21% (based on an estimate

of 50% G$_0$ fertility). Several transformant lines were verified by Southern hybridization and sequencing of internal fragments and genomic insertion sites.

In order to select lines having a stabilized marker for potential field release in SIT programmes, three transformed lines expressing both markers (and thus subject to stabilization; see below) were tested for several quality control parameters previous to stabilization. These tests assessed viability at all life stages, fertility, adult flight ability and adult male sexual competitiveness. All of the lines were less fit compared with the untransformed wild-type strain for the first three parameters by approximately 5–10%; however, no significant difference was found in any of the lines for male sexual competitiveness. This result is encouraging for the use of transgenic mexflies in field release programmes, since effective mating competitiveness is considered to be the major prerequisite for male release (Orozco-Davila *et al.*, 2007).

The vector for spermatocyte-specific marking, pBXL[*PUbEGFP/Asß2t-DsRed.T3*] was also transformed into mexfly with the phsp-pBac helper by injection into embryos, yielding 38 G$_0$ adults that were mated in 11 small groups resulting in putative G$_1$ transformants from five of the groups at an estimated frequency of approximately 13%. These transformant lines were inspected for whole-body EGFP fluorescence as well as testis- and sperm-specific DsRed fluorescence, but did not undergo further molecular analysis. Nevertheless, one of the transformant lines appeared to have a Y-linked vector integration based upon male-specific expression of the PUb-nls-EGFP marker. But curiously, sperm-specific DsRed fluorescence was not apparent in any of the male transformants. To determine if the *β2-tubulin* promoter was subject to position effect suppression, the vector insertion was re-mobilized by injection of the transposase helper plasmid (J.S. Meza and A.M. Handler, unpublished). New Y-linked insertions having sperm-specific *DsRed* expression could not be identified, but at least one new autosomal insertion, based on *PUb-nls-EGFP* expression in both males and females, also exhibited restoration of the *Asß2t-DsRed.T3* marker expression. This suggests the

possibility for some type of Y-specific suppression of the male-specific gene promoter that warrants further investigation.

21.3.5   *piggyBac* transformation of the Queensland fruit fly, *Bactrocera tryoni*

The Queensland fruit fly, *Bactrocera tryoni*, is the major pest of fruit production in Australia, and, similar to other tephritid pests, is controlled primarily by SIT. *B. tryoni* was transformed with two *piggyBac* vectors, pB[*PUb-DsRed1*] (Handler and Harrell, 2001a) and pB[*PUbnlsEGFP*] (Handler and Harrell, 1999, 2001b), in a series of experiments where either plasmid vector was co-injected with the phsp-pBac helper (Raphael *et al.*, 2011). From embryos injected with the DsRed vector, 71 G$_0$ adults survived that were backcrossed to non-transformed flies yielding G$_1$ transformants from five groups, while embryos injected with the EGFP vector yielded 61 G$_0$ adults and G$_1$ transformants from two groups. Minimum transformation frequencies from these experiments were estimated to be 5.3% to 10.3%, respectively. *piggyBac*-mediated transformation was verified in each line by internal PCR to the marker coding regions and in four lines where the insertion sites were sequenced by inverse PCR. Similar to *B. dorsalis*, PCR sequencing also identified endogenous sequences nearly identical to the IFP2 *piggyBac* element, extending the range of *piggyBac* in *Bactrocera* beyond the *B. dorsalis* complex.

This study represented the first transposon-mediated transformation of the Queensland fruit fly; although a prior attempt using a *hobo* vector yielded transformants, these were due to imperfect transposition events (possibly recombinant) that were eventually unstable (S. Whyard, unpublished; see Raphael *et al.*, 2004).

21.3.6   *piggyBac* transformation of the olive fruit fly, *Bactrocera oleae*

The first *piggyBac*-mediated transformation of the olive fly had the goal of creating conditional

female-lethal strains using the RIDL system (Release of Insects carrying a Dominant Lethal; see section 21.9.2, below) (see also Scott *et al.*, Chapter 17; Morrison, Chapter 23, this volume) (Ant *et al.*, 2012). For this strain a vector, OX3097, was created that incorporated a fluorescent protein marker (*hr5-IE1-DsRed2*), and a tetracycline transcriptional activator (tTAV) that was female-specifically expressed by the upstream insertion of the sex-specifically spliced first intron from the medfly *transformer* (*Cctra*) gene (Pane *et al.*, 2002; Fu *et al.*, 2007). In the absence of tetracycline, this resulted in the female-specific production of tTAV that reaches toxic levels in late larvae and pupae. From 4500 embryos injected with the OX3097 plasmid vector and *piggyBac* mRNA, 138 G_0 adults survived that were backcrossed to wild-type flies in small groups. This yielded six G_1 transformant lines at an approximate frequency of 4%. These lines were apparently tested for *piggyBac*-mediated integration by insertion site sequencing (data not presented). To assess the efficacy of these lines for use in SIT, mating competitiveness, mating synchronicity and the propensity of wild females (previously mated to transgenic males) to re-mate were tested, with supportive results. Importantly, weekly releases of transgenic males into caged wild-type olive fly populations resulted in a population decline, presumably due to increasing female lethality.

21.4 Transformation with the *Hermes* Vector System

The third transposon vector system to transform medfly was the *hAT* (*hobo*, *Activator*, *Tam3*) superfamily element, *Hermes*. *Hermes* was isolated from the housefly *Musca domestica* by degenerate PCR using primers based upon common amino acid motifs from other *hAT* elements (Warren *et al.*, 1994) (see O'Brochta, Chapter 1, this volume). Previous to testing in medfly, *Hermes* was already established as an effective transformation vector system in a wide range of insects, including *D. melanogaster*, *Aedes aegypti*, *Culex*

quinquefasciatus, *Stomoxys calcitrans* and *Tribolium castaneum* (Handler and O'Brochta, 2012). For testing in medfly, the *Hermes* vector, pH[*hsp70Ccw*], was marked with the *hsp70*-regulated medfly *white eye*+ gene, similar to the previous *Minos* and *piggyBac* vectors created for this species (Michel *et al.*, 2001). From more than 2500 injected embryos, 186 surviving G_0 adults were pool-mated, resulting in one pigmented red eye G_1, yielding a relatively low transformation frequency of less than 1%. But as later determined in *Drosophila*, the *white*+ marker is not highly reliable, due to sensitivity to genomic position effects (Handler and Harrell, 1999), and not all G_1 transformants may have been detected. *Hermes*-mediated transformation was verified by Southern hybridization, polytene chromosomal *in situ* hybridization and inverse PCR, but these analyses also indicated that two independent single integrations occurred in the various G_1 lines. Indeed, unlike the other medfly transformations, some of the G_2 transformants displayed a lighter yellow-eye phenotype compared with the parental G_1 red-eye transformants.

21.5 Transformation with the *hopper* Vector System

In an effort to identify *hAT*-related transposons in tephritid species, degenerate primers to conserved amino acid sequence motifs were used for PCR in several tephritids (Handler and Gomez, 1996). A nucleotide sequence was obtained from the oriental fruit fly *B. dorsalis*, conceptually translated into a 144 amino acid *hAT*-related sequence, and used to screen genomic clones for complete transposons from a Kahuku strain lambda GEM12 library (Handler and Gomez, 1997). One positive clone, named *hopper*[Bd-Kah], was found to include a 3120 bp sequence with 19 bp terminal inverted repeat (TIR) sequences with a single mismatch. Internal to the TIRs was a 1.9 kb consensus transcriptional unit encoding a *hAT*-related transposase including multiple mutations and frameshifts indicating a non-functional

hAT-related transposable element. Genomic Southern blots to several *B. dorsalis* strains and the closely related melon fly, *Zeugodacus cucurbitae*, showed the presence of highly conserved elements. Using *hopper*[Bd-Kah] sequence primers, PCR screens in these strains resulted in the discovery of a new 3131 bp element, *hopper*[Bd-we], in the *B. dorsalis white eye* strain (Handler, 2003). Unlike *hopper*[Bd-Kah], *hopper*[Bd-we] had an uninterrupted 1950 bp reading frame and other attributes consistent with transposon function, which was tested by creating two transformation vector plasmids and a *D. melanogaster hsp70*-regulated helper (Handler and Schetelig, 2020). A pKhop[*Dmwhite*+] vector was used in a mutant-rescue transformation of the *D. melanogaster white*– strain, and a phop[*PUbDsRed.T3*] red fluorescent-marked vector was tested in both *D. melanogaster* and *A. suspensa*. Transformants were selected in all three experiments, but transformation frequencies were relatively low at 1.6% and 2.5% per fertile G_0 in *Drosophila* and about 10% in *Anastrepha*. One interesting finding was that two of the four *Drosophila* transformations were X-linked recessive lethal insertions, based on segregation analysis, and one of the nine *Anastrepha* transformations was a putative Y-linked insertion, indicating a possible bias for sex-linked chromosome insertions.

21.6 Marker System for Transformant Organismal and Tissue Detection in Tephritid Flies

As noted for several of the first germline transformation experiments in tephritid species, and mosquitoes as well, mutant-rescue markers for visible mutations were used, as they had been used routinely in *Drosophila*. These were predominantly for eye colour mutations, where the marker was a wild-type clone of the mutant gene, with the expectation that a wild-type phenotype would occur by complementation in transformed host individuals. For efficient transformation systems, mutant rescue markers are highly useful, but for most non-drosophilid

insects the requisite visible mutation strains do not exist, nor are the cloned wild-type genes necessary for rescue. This made the development of new widely applicable dominant-acting markers a high priority. A list of the first uses of a transposon vector with a specific marker in an insect species is shown in Table 21.1.

21.6.1 Transformant marking systems

Chemical resistance markers

Several attempts were made to develop drug and insecticide resistance markers, such as neomycin phosphotransferase II (NPTII) and organophosphate dehydrogenase (*opd*). NPTII, providing resistance to the neomycin analogue G418, was effective in *Drosophila* (Steller and Pirrotta, 1985), but resulted in false-positive survivors when used to test *P*-element vectors (pUChsneo) in *A. suspensa*. This may have been the result of neomycin (kan[r]) resistant bacterial colonies found to be present in the gut and sub-oesophageal bulbs (A.M.H. and S.P. Gomez, unpublished). The *opd* gene provides resistance to toxins such as parathion, as shown in *Drosophila* when tested as a transformation selection marker (Benedict *et al.*, 1995), but we found low consistent levels of resistance to this toxin in control wild-type caribflies, making tests for transformant selection unreliable. While none of the drug resistance marking systems were found to be successful in tephritid species, the few that were tested in transformation experiments used vector systems, such as *P*, that were never proven to be functional. Although highly effective fluorescent proteins markers now exist, chemical resistance markers have the advantage of allowing transformant selections *en masse*, which can be highly useful for species that cannot be transformed efficiently. It might now be possible to test chemical resistance markers along with fluorescent protein (FP) markers that verify transformation, to better evaluate, and possibly improve, efficacy of the chemical resistance.

Fluorescent protein gene markers

One of the first FP gene markers was the native *GFP* gene (Chalfie *et al*., 1994) linked to a *D. melanogaster polyubiquitin* promoter (Lee *et al*., 1988) and a nuclear localizing sequence (nls) to observe nuclear accumulation of GFP in *Drosophila P* vector transformants (Davis *et al*., 1995). Beyond its use in *Drosophila* to regulate the first successful FP marker system, the *polyubiquitin* promoter proved to be a fortuitous choice for FP markers in other species and, in particular, tephritid flies. This was due to the highly conserved structure and function of the *polyubiquitin* gene, known to be constitutively expressed in all tissues throughout development as part of the proteasome–ubiquitin pathway for non-lysosomal proteolysis (Lee *et al*., 1988). The *Drosophila* promoter has retained function in all tephritid species tested, and its promotion of whole-body FP expression has made it valuable in identifying insects trapped in the field.

We modified the marker by exchanging *GFP* for the enhanced-*GFP* (*EGFP*) variant, which was first tested using a *piggyBac* vector with a mini-*w*⁺ secondary marker pB[*w*⁺, *PUb-nls-EGFP*] in *Drosophila*. The pB[-*PUb-nls-EGFP*] vector was then tested in *A. suspensa*, showing that EGFP was highly expressed and effectively regulated by the *Drosophila* promoter. Importantly, the dual-marked vector in *Drosophila* produced several transgenic lines that expressed EGFP, but not visually identifiable pigmented eyes. A similar result occurred in *B. dorsalis*, indicating that EGFP and probably other FPs would be more reliable for transformant marking than the *white*⁺ gene or its cognates, due to their sensitivity to genomic position effects.

Similar to *polyubiquitin*-regulated *EGFP*, the *DsRed* fluorescent protein was linked to the constitutive promoter (Handler and Harrell, 2001a) revealing highly intense fluorescent expression in all species tested, which in some cases could be detected in the late embryonic and larval stages (Schetelig and Handler, 2013b). Other promoters for transformant marking include the baculovirus promoters *Achr5-IE1* and *OpIE2* (Morrison

et al., 2010). Notably, the *3xP3* artificial promoter linked to various FPs (Horn *et al*., 2002), which was widely used in several dipterans and in three other insect orders, was not successful as a visible marker for transformants in medfly, mexfly or caribfly (A.M.H., unpublished). In caribfly, we also tested a vector dual-marked with *PUb-nls-EGFP* and *3xP3-AmCyan* that only expressed whole-body green fluorescence (Schetelig and Handler, 2012b), while use of the same vector in *Drosophila* resulted in expression of both markers (Schetelig and Handler, 2013a). Transcript analysis by qPCR later showed the unique failure of *3xP3* promoter function in the tephritid, which is highly unusual and suggests a difference in *eyeless/ Pax-6* function between tephritids and other insect species. Regardless of the basis of this difference, the inability to use *3xP3*-regulated markers for tissue-specific expression is an important impediment for transgenic strain development in tephritid species. This is due to most other available promoters being constitutive, which is problematic for the use of multiple FPs that cannot be easily distinguished when co-expressed.

Fluorescent protein markers for field detection

A critical component of SIT is the ability to monitor released males, and the ability to distinguish them from those in the field population when collected in traps. Soon after FPs were used for transformant selection, their use was also considered for the field detection of transgenic males collected in traps. This was reinforced by the stability of GFP and DsRed fluorescence, for up to 2–3 weeks, in dead flies kept under dry conditions (Handler and Harrell, 2001b), yet it was unknown whether fluorescence could be reliably detected in flies collected in liquid traps. This was tested by monitoring DsRed fluorescence in transgenic caribflies maintained in torula yeast borax (TYB) or propylene glycol (PG) solutions that are commonly used in insect traps (Nirmala *et al*., 2011). Under both trap conditions fluorescent flies could be reliably distinguished from

non-transformed flies for up to 3 weeks, the usefulness of which was reinforced by the PCR detection of a 130 bp fragment within the marker gene.

21.6.2 Spermatocyte-specific transgene marking

A high priority for SIT programmes has been the ability to determine if trapped wild females in the field had mated to released sterile males. Although efforts were made to distinguish sperm from released males and those in the field by differences in sperm length from irradiated and non-irradiated males (McInnis, 1993), this method is neither simple nor unambiguous. A more straightforward identification of sperm within females that had mated with transgenic males became apparent from sperm-labelling experiments in mosquitoes. In both *Anopheles stephensi* and *Aedes aegypti*, promoters from their endogenous spermatocyte-specific *β2-tubulin* genes were linked to *EGFP* and *DsRed*, respectively, to generate fluorescently labelled sperm (Catteruccia *et al*., 2005; Smith *et al*., 2007) (see Nolan and Hammond, Chapter 3, this volume). For these species, which are not currently controlled by SIT, sperm fluorescent marking makes it possible to separate males from females during larval development and clearly identify the testes and individual sperm in adults (Marois *et al*., 2012). For use in tephritid species, endogenous *β2-tubulin* genes were similarly isolated and linked to turbo-GFP/EGFP or DsRed variants, and transformed into the medfly, caribfly and mexfly (Scolari *et al*., 2008; Zimowska *et al*., 2009; Meza *et al*., 2011). For all three species, fluorescent sperm could be unambiguously identified after extrusion from male testes and the spermathecae in non-transgenic females mated to transgenic males. In the caribfly studies, transgenic sperm could also be detected by PCR on DNA extracted from female abdomens, providing a molecular verification for the visible phenotype (Zimowska *et al*., 2009). In contrast to the mosquito studies, however, fluorescence in the medfly

and caribfly testes was only occasionally detectable at the last larval stage, making the system unreliable for sorting sexes at larval stages. Nevertheless, the ability to rapidly identify sperm from transgenic males in mated females provides a major improvement for monitoring tephritid SIT programmes.

21.6.3 Y-linked vector integrations for male-specific marking

In *D. melanogaster*, transposon vector integrations on the Y chromosome are relatively rare, presumably due to its relatively small size and, potentially, the position effect suppression of the often-used w^+ eye colour marker by heterochromatin, or biases for insertion sites by the commonly used *P* element vector (see section 21.6.1). Y-chromosome insertions appear to be more prevalent in tephritid species (typically based on male-specific expression, and not cytological localization), potentially due to greater amounts of Y-specific euchromatin, use of FP markers less susceptible to position effect suppression, or the use of vector systems that are less restricted with respect to insertion site specificities. Y-linked integrations have been reported in medfly using *piggyBac* vectors marked with either *DsRed2* or *ZsYellow* (Condon *et al*., 2007b), and a single Y-linked insertion in mexfly using a vector marked with *PUb-nls-EGFP* and the sperm-specific *Asß2t-DsRed.T3* marker (Meza *et al*., 2011; Meza *et al*. 2014b) (see section 21.3.4). We have also noted male-specific insertions in at least six lines in caribfly, with one line having late embryonic expression of the *PUb-DsRed* marker (Schetelig and Handler, 2013b). An obvious use for these transgenic lines is sorting the sexes for male releases in SIT using automated fluorescent sorters. These systems are already available for selecting fluorescent mosquitoes or *Drosophila* embryos and early-instar larvae (Furlong *et al*., 2001; Marois *et al*., 2012) and should be suitable for sorting by early developmental FP marker expression of the Y-linked line in caribfly. Potentially, Y-linked translocations

could also be created with autosomal chromosomes having FP insertions that are known to express in embryos (e.g. caribfly lines transformed with pB[*PUbDsRed.T3-AsProsβ2[1]*]; see section 21.9.1 and Nirmala *et al.*, 2009).

21.7 Post-integration Stabilization of Transposon Vectors in Tephritid Flies

The effective use of transgenic strains for control programmes will depend on the reliable expression of the integrated genes of interest and the maintenance of strain fitness and viability under mass-rearing protocols. Transgene vectors must be stably integrated to maintain strain integrity and prevent possible interspecies movement of the transgene into unintended hosts, which is a major concern for ecological safety (Hoy, 2003; Handler, 2004) (see Hayes and Quinlan, Chapter 28, this volume). The major likely contributing factor to vector instability is the unintended (or unknown) presence of transposase from the same transposon, or a functionally related system in the host genome or in an associated infectious or symbiotic organism within the host. The former possibility can be tested directly by molecular identification of the same or closely related element with a high degree of sequence identity (Handler and McCombs, 2000; Handler, 2004). However, related systems may be functionally conserved but lacking sufficient structural identity for easy detection, as would be the case for *hobo* and *Hermes* that have the ability to cross-mobilize one another (Sundararajan *et al.*, 1999). While transposition and excision mobility assays for the vector transposon may be performed in the host organism (embryos or cell lines) with and without transposase helper to assess this possibility, these assays are probably not sensitive enough to detect mobility catalysed by transposase from co-existing organisms that proliferate post-embryogenesis. Given these caveats, it is unlikely that the complete potential for transgene vector remobilization can be definitively and unambiguously assessed for each potential host insect, presenting the possibility that

any transgenic insertion may be remobilized at some time and thus raising concern for the ecological risks for transgenic release (Young *et al.*, 2000; FAO/IAEA, 2006).

The concern for potential remobilization of a transgene vector by the unintended presence of the vector transposase, or a related cross-mobilizing transposase, is most directly addressed by the deletion or rearrangement of the transposon ITR sequences required for transposition. One approach tested in *Drosophila* was recombination between single *FRT* recombination sites within two independent *piggyBac* vectors integrated in the same chromosome (Handler *et al.*, 2004; Schetelig *et al.*, 2011a). This exchanged the 5′ ITR from one vector with the 3′ ITR from the other, thus immobilizing both vectors, which had only one ITR or the other. Despite its elegance, this approach is technically difficult, most easily achieved in genomes that have been sequenced, and is currently too formidable for routine use in other insects.

21.7.1 Vector stabilization by post-integration deletion of a single terminal sequence

To stabilize transposon vectors subsequent to genomic integration, we took an alternative approach that was initially tested in *Drosophila* (Handler *et al.*, 2004) and then implemented in the caribfly, medfly and mexfly, but which is simply applicable to all species subject to transposon-mediated transformation. This was achieved in *Drosophila* (Handler *et al.*, 2004) by introducing an internal tandem duplication of one of the terminal sequences, with genes of interest (GOI) placed between the duplicated ITRs. After integration, the internal ITR could be remobilized with the non-duplicated ITR, leaving the duplicated external ITR and proximal GOI genomically integrated. With only a single terminal sequence, the remaining transgene was found to be stable with respect to the vector transposase. Specifically, for testing in *Drosophila* a *piggyBac* stabilization vector, pBac{L1-PUbDsRed1-L2-3xP3-ECFP-R1}, was created by placing a duplicated 5′ ITR (pBacL2) internal to the flanking 5′ (pBacL1)

and 3′ (pBacR1) ITRs, with a PUbDsRed1 marker placed between L1 and L2, and a 3xP3-ECFP marker placed between L2 and R1. In a transformant line having the complete vector integration (exhibiting both marker phenotypes), the L2-3xP3-ECFP-R1 'sub-vector' was deleted by remobilization after mating to a *piggyBac* transposase jump-starter strain (having a chromosomal transposase gene). Red fluorescent progeny had only the L1-PUbDsRed1 transgene sequence remaining, which was expected to be stable in the absence of the R1 3′ *piggyBac* terminus. This was tested by mating the L1-PUbDsRed1 line to the jump-starter strain, which showed that remobilization of the remaining transgene did not occur (by loss of phenotype) in more than 7000 progeny assayed, compared with a remobilization rate of about 5% in the original L1-PUbDsRed1-L2-3xP3-ECFP-R1 vector. This showed that the L1-PUbDsRed1 transgene was stabilized owing to the loss of the 3′ *piggyBac* terminus.

We subsequently created modified stabilization vectors for tephritid species and stabilized them in *C. capitata* (Schetelig *et al.*, 2009b), *A. ludens* (Meza *et al.*, 2011) and *A. suspensa* (A.M.H., unpublished). These vectors were efficiently stabilized by mating to a jump-starter strain in the medfly or injecting a transposase helper plasmid in *Anastrepha* species, which simplifies the process for many insects not having a jump-starter strain. In medfly, vector stability was tested quantitatively where, after mating to a jump-starter strain, the transgene remained stably integrated in over 70,000 progeny screened for loss of the marker. A stabilization vector was similarly integrated into the olive fruit fly, *B. oleae*, by *piggyBac* transformation (Genc *et al.*, 2016), though vector stability after sub-vector remobilization has yet to be evaluated.

21.7.2 Vector stabilization by deletion of both terminal sequences

Subsequent to development of stabilization by single ITR deletion, a new method was tested with *piggyBac* that deleted all transposon vector sequences including the 5′ and 3′ ITRs (Dafa'alla *et al.*, 2006). To achieve this a vector was created that consisted of two functional transposon vectors, in tandem orientation, with both having 5′ and 3′ ITRs with a marker gene insertion. The two vectors were separated by a spacer region having another marker sequence, and where genes of interest for stabilized integration would be located. Upon transformation, one possible integration event occurred that included both vector and spacer sequences, using the 5′ ITR of the first vector and the 3′ ITR of the second vector. When this transformant line, expressing all three markers, was re-exposed to transposase, the flanking vector sequences were excised together or sequentially, resulting in the stabilized integration consisting of the single marker and spacer sequences. While this dual terminus deletion system worked reasonably efficiently, it is considerably more complex than the single ITR deletion approach, and its practical advantage remains to be evaluated.

21.8 Site-Specific Genomic Targeting in Tephritids

All of the transposons used for transformation vectors integrate randomly in genomes except for short nucleotide sequence specificity (e.g. TTAA for *piggyBac* and TA for *Minos* and *mariner*), or general biases for genomic regions or gene structures that are not well understood (e.g. *P* integrations in 5′ regulatory sequences) (Thibault *et al.*, 2004). These insertions may cause mutations due to disruption of coding or regulatory sequences that are useful for insertional mutagenesis strategies, but are a significant drawback to the development of transgenic strains for applied use when fitness costs result (Catteruccia *et al.*, 2003; Irvin *et al.*, 2004). Random transgene integrations also result in variable transgene expression resulting from chromosomal position or enhancer effects (Schotta *et al.*, 2003). Position effect transgene suppression can be controlled to some extent by placement of proximal insulator elements (Sarkar *et al.*, 2006), but their effectiveness varies and repetitive

insulator sequences may be subject to *cis*-recombination resulting in GOI deletions (Zhao *et al*., 2020). Transgene insertions are affected by nearby promoters and enhancers or other epigenetic influences resulting in altered expression with respect to developmental and tissue specificities. Thus, the random nature of most transposon vector insertions creates a great challenge for transgenic strain development, which can be addressed most directly by the development of site-specific genomic targeting strategies.

21.8.1 Recombinase-mediated cassette exchange

A recombinase-mediated cassette exchange (RMCE) targeting system (Baer and Bode, 2001) using the *FRT*/FLP recombination system (Andrews *et al*., 1986; Siegal and Hartl, 1996) was first tested in *Drosophila* (Horn and Handler, 2005). This was based upon an acceptor target vector that, once genomically integrated by *piggyBac* transformation, could act as a target site for subsequent donor vector insertions by double recombination of hetero-specific *FRT* sites that have sequence variations within their 8 bp variable core region. Since only identical (i.e., homo-specific) sites can recombine with one another in the presence of recombinase, the *FRT* variant sites within the donor and acceptor vectors cannot recombine with one another (resulting in a deletion or inversion of intervening sequences). But they can recombine with the same homo-specific sites present within the two vectors, resulting in an exchange of the respective intervening sequences by double recombination. Thus, an RMCE-acceptor vector, pBac[*3xP3-FRT-ECFP-linotte-FRT3*], was created having hetero-specific *FRT* and *FRT3* sites in tandem orientation, which flank the *ECFP* marker coding region and the *Drosophila linotte* gene 'homing' sequence (which acts as a 'bait' for genomic *linotte* sequences by para-homologous pairing). The *FRT* site was placed between the *3xP3* promoter and *ECFP*. The original RMCE donor plasmid, pSL-*FRT-EYFP-linotte-FRT3*, is devoid of *piggyBac* sequence and comprises a donor

cassette having the *FRT* and *FRT3* sites flanking a promoter-free *EYFP* coding region and the *linotte* homing sequence. After co-injection with FLP recombinase helper plasmid into homozygous transformant acceptor vector lines, targeted cassette replacements were identified in progeny by eye-specific EYFP expression (in place of ECFP). By using the 3xP3 promoter, within the target site, for expression of the exchanged EYFP cassette, identification of RMCE events was ensured (versus non-targeted insertions). A subsequent donor vector tested in *Drosophila* incorporated a *piggyBac* 3′-ITR (pBacR1) sequence linked to a *PUb-DsRed* marker that, once integrated, could be remobilized with the acceptor vector *piggyBac* 5′-ITR, resulting in genomic stabilization of the original acceptor vector *piggyBac* 3′ sequence and associated genes of interest (see above).

The described *FRT*/FLP RMCE system has yet to be successfully tested in a non-drosophilid species, but a similar RMCE system using the Cre/*loxP* system was also created for use in *Drosophila* with a *P*-element target site vector and eye and body colour mutations to mark recombinants (Oberstein *et al*., 2005). New Cre/*lox* RMCE systems were successfully created and tested in *D. melanogaster*, *D. suzukii* and *A. suspensa*, using a *piggyBac* target site vector and the markers *PUb-EGFP, PUb-DsRed* and *3xP3-ECFP* (Schetelig and Handler, 2013a; Schetelig *et al*., 2019). In the caribfly, specifically, this was used to test *3xP3* promoter function using RMCE to control for the possibility of position effect suppression, by expressing PUb-DsRed recombined into the same genomic insertion-site position (see section 21.6.1).

The use of gene targeting in tephritids, and many other species, would most likely start by creating several target sites in the genome of each species, with host strains tested for mutational and position effects that might negatively affect life fitness, fecundity, mating competitiveness and transgene expression, among other parameters. Optimal strains would then be used for further development by targeting desired transgene cassettes into the target sites,

such as a transposon stabilization cassette that was demonstrated in *Drosophila* (Horn and Handler, 2005). Any introduced cassette could be removed by exchange, and the number of new insertions would only be limited by the number of new hetero-specific recombination site pairs that can be introduced.

21.8.2   phiC31-mediated recombination

A phiC31 unidirectional system to site-specifically integrate DNA at specific target sites was also tested and used in several studies in *D. melanogaster* (Groth *et al.*, 2004; Venken *et al.*, 2006; Bischof *et al.*, 2007; Venken and Bellen, 2012). It has since been successfully tested in two mosquito species, *Ae. aegypti* and *Aedes albopictus* (Nimmo *et al.*, 2006; Labbé *et al.*, 2010), and the tephritid species *C. capitata* (Schetelig *et al.*, 2009b) and *A. ludens* (Meza *et al.*, 2014a). Integrations require two attachment sites, *attP* and *attB*, that recombine with each other in the presence of the phiC31 integrase enzyme (see Ahmed and Wimmer, Chapter 5, this volume). This results in the integration of *attB*-containing plasmid DNA into a genomic *attP* landing site that creates two new attachment sites, *attR* and *attL*, flanking the integrated plasmid. However, unlike RMCE this process is irreversible, since the integrase cannot catalyse excision from the *attR* and *attL* sites, or a new integration into them.

For testing in medfly, the phiC31 integrase system was used to modify previously inserted transgenes by site-specific integration (Schetelig *et al.*, 2009b). Thus, suitable transgenic strains carrying a single copy of an *attP* landing site were first generated by *piggyBac*-mediated germline transformation. In a second step, donor plasmids containing an *attB* site, additional markers and transposon ends were integrated into the *attP* sites by phiC31 integrase-mediated recombination. The resulting strains containing two fluorescent markers for visualization and three transposon ends were then mated to a *piggyBac* jump-starter strain, resulting in post-integration excision of a marker and two transposon ends for stabilization (see

section 21.7.1). This three-step integration and stabilization system allows the combination of several advantageous transgene-encoded traits at evaluated genomic positions to generate optimized strains for eventual release.

While genomic targeting via site-specific recombination adds versatility to the manipulation of insect genomes, a critical question is whether successive insertions into specific genomic sites have an effect on transgene expression or on host insect fitness. A possible effect on host fitness was addressed for two *A. ludens* strains that were originally transformed with a *piggyBac* vector carrying an *attP* site (Meza *et al.*, 2014b). Both transgenic strains retained fitness parameters similar to wild-type, which was then compared after a secondary phiC31-mediated insertion of an *attB-EGFP* donor vector into the *attP* target site. Fitness test comparisons of the dual-insertion strains to their single-insertion predecessors showed no significant difference in one strain, while the second strain exhibited significantly lower fitness parameters. The basis for the change in fitness for one strain is unknown but, regardless, at least for secondary phiC31-mediated insertions it cannot be assumed that fitness parameters will remain unchanged and therefore additional evaluation will be required.

21.9   Transgenic Strains for Improved Population Control of Tephritids

The relative ease of transforming tephritids has given added impetus to creating transgenic strains in several species to improve biologically based control programmes, SIT in particular. The development of organismal and spermatocyte-specific marking systems for improved SIT has been discussed and, in addition, several approaches have been taken to improve sexing and male sterility using dominant-acting conditional lethal gene expression. The rationale for this approach in terms of sterility is that males (and females if viable) can be reared under

permissive conditions that allow their survival to adulthood and, once released, their progeny should die in early development in the field under non-permissive conditions. This approach is actually a type of genetic sterility, where released males fail to produce fertile offspring, though a similar approach may be possible for tissue-specific gonadal sterility. Similarly, for sexing, both males and females would survive under permissive conditions for rearing, but female-specific expression of the lethal gene under non-permissive conditions should allow only males to survive for male-only releases. Two types of conditional lethality have been tested in tephritid flies: one based on a temperature-sensitive mutation that causes death at elevated temperatures; and a tetracycline-dependent system where lethality is suppressed by dietary antibiotic.

21.9.1 Conditional lethality using a dominant temperature-sensitive mutation

One approach towards conditional lethality uses a dominant temperature-sensitive (DTS) mutation in the proteasome subunit gene, *Prosβ2*, first described in *D. melanogaster* as DTS-7 (Smyth and Belote, 1999). This is a missense mutation in the 20S subunit that results in a DTS 'poison subunit' or antimorph that disrupts proteasome function, resulting in late larval or pupal death in insects reared at 29°C. For SIT, a DTS strain could be reared at 25°C or below and released into the field in tropical regions, with progeny failing to survive at ambient temperatures of 29°C or above. To test this system for use in tephritid flies for genetic sterility, the highly conserved *A. suspensa* cognate of *Prosβ2* (*AsProsβ2*) was isolated by degenerate PCR and then mutated to the *AsProsβ2[1]* DTS allele by *in vitro* mutagenesis (Nirmala *et al.*, 2009). After transformation into a wild-type caribfly host strain, four transgenic lines homozygous for the mutant transgene developed into pupae at similar frequencies at both 25°C and 29°C, but failed to eclose as adults at rates of 96–100% at 29°C. These results demonstrated that *AsProsβ2[1]* has an effective

dominant temperature-sensitive phenotype, but lethality was focused on the pupal stage, which is less than optimal for a larval pest. The possibility of modifying this system for female-specific lethality has not been pursued.

21.9.2 Conditional lethality using a tetracycline-suppressible (Tet-Off) lethal system

A different conditional lethal approach towards improving SIT is by use of the tetracycline-controlled transcriptional activation system from *E. coli* developed for use in mammalian systems (Gossen and Bujard, 1992). The tetracycline-suppressible (Tet-Off) binary system was also used to control gene expression in *Drosophila* (Bello *et al.*, 1998) and later tested in this species by several laboratories as a model for embryonic and female-specific lethality for improved SIT by suppressing lethal gene function with dietary tetracycline (Tet) (Heinrich and Scott, 2000; Thomas *et al.*, 2000; Horn and Wimmer, 2003) (see also Scott *et al.*, Chapter 17; Morrison, Chapter 23, this volume). The Tet-Off system comprises a tet-transactivator (*tTA*), whose expression is regulated by a defined promoter, which acts in *trans* by binding to a Tet response element (TRE) (consisting of multiple Tet operator (*tetO*) sequences) to promote expression of a linked coding sequence. The tTA can also bind to tetracycline and its analogues, which inhibits its binding to the TRE, thus preventing expression of the coding sequence. In this way, the spatial, temporal and sex-specific expression of a gene of interest, such as a lethal gene, may be regulated by the *tTA*-linked promoter and can be suppressed by the presence of tetracycline. The binary Tet-Off systems in insects are typically created by the mating of independent strains created for the tTA driver lines and the TRE effector lines, to test for optimal expression of each component (that might be compromised by position effects) and to use them interchangeably. For lethality or sterility systems the components could be maintained

together in the same strain, though constant rearing on Tet would be required.

21.9.3   The release of insects carrying a dominant lethal (RIDL) system

RIDL® is based on a Tet-Off lethal system that renders transgenic males (reared on Tet) genetically sterile due to their progeny failing to survive in the absence of Tet (Thomas *et al.*, 2000; Gong *et al.*, 2005). This system and a modified female-specific lethality system for genetic sexing (Fu *et al.*, 2007) is discussed in detail by Morrison, Chapter 23, this volume. In summary, and in contrast to the Tet-Off system used for embryonic lethality described below, the current RIDL system tested in medfly comprises the TRE linked to the *tTA* (modified to a tTAV), resulting in a self-regulated system where tTA drives its own expression (Gong *et al.*, 2005). Accumulation of tTA during development eventually reaches toxic levels in the absence of Tet, resulting in death by late larval or pupal stages. It is expected that when males carrying the *TRE-tTAV* transgene reared on Tet-diet are released to the field, their progeny will die due to tTA toxicity. A modification of this system tested in medfly (Fu *et al.*, 2007) and the olive fly (Ant *et al.*, 2012) for female-specific lethality has the sex-specifically spliced first intron of the medfly *transformer* gene inserted upstream of the tTAV coding sequence, allowing tTAV to be expressed only in females, resulting in a males-only line. The impact of a genetically engineered olive fly strain on three non-target organisms that either predate or parasitize olive flies was subsequently evaluated, with no significant negative effect reported on life history parameters, mortality and reproductive capacity of the non-target organisms studied (Marubbi *et al.*, 2017). A recent population genetics model with density dependence to evaluate the relative effectiveness of female-killing approaches based on single- and two-construct arrangements was conducted (Vella *et al.*, 2021). This study suggested that a single-construct RIDL arrangement resulted in slightly faster population

suppression, but the two-construct arrangements (for examples, see section 21.9.4) can eventually cause stronger suppression and cause local population elimination with a smaller release size.

21.9.4   Conditional embryonic lethality using a Tet-Off lethal system

A Tet-Off conditional lethality system resulting in embryonic death was first developed in *Drosophila* by linking the early embryonic *serendipity α* (*sry-α*) promoter to the *tTA*, which induced lethality by promoting expression of a *hid* pro-apoptotic cell death gene variant (*hid*[Ala5]) linked to the TRE (Horn and Wimmer, 2003). The ability to achieve total embryonic lethality in several driver-lethal effector strains, effectively suppressed by Tet, encouraged the testing of this system in tephritid species. This was first achieved in medfly, where the *D. melanogaster hid*[Ala5] was effective, but expression of *tTA* controlled by *Dm-sry-α* was not (Schetelig *et al.*, 2007, 2008, 2009a). Therefore, the medfly cognate (*Ccsry-α*) for *Dmsry-α* was isolated and a driver strain was constructed using its promoter to regulate the *tTA*. Crosses between *Ccsryα-tTA* driver and TRE-*Dmhid*[Ala5] effector strains resulted in two driver–effector strains that were 100% lethal in the absence of Tet-diet, though this required 60 driver–effector line matings, presumably due to most lines having diminished expression resulting from position effects.

To transfer the Tet-Off embryonic lethality system to other tephritids, similar transgenic driver and effector lines were created for the caribfly, but the constructs for these lines were pre-evaluated by qPCR and functional *in vitro* cell death assays to determine which driver and effector constructs were most efficient (Schetelig and Handler, 2012a). Since preliminary tests suggested that the *Ccsry-α* promoter might have diminished function in *Anastrepha*, the native *Assry-α* promoter was isolated from *A. suspensa* to create *Assryα-tTA* embryonic driver strains that were evaluated by qPCR, showing that two of five strains tested efficiently

promoted tTA expression. While the *Dmhid*[Ala5] lethal effector was effective in medfly, 12 effector lines had to be tested to identify one that yielded 100% lethality with two driver lines. This suggested that a native cell death gene would be more effective, which motivated the isolation of the nearly identical *hid* orthologues from *A. suspensa* and *A. ludens* (Schetelig *et al.*, 2011b; Schetelig and Handler, 2012a). Testing *A. ludens hid* and its phospho-mutated variant *Alhid*[Ala2] (which prevents inhibitory phosphorylation) in *in vitro* cell death assays did, indeed, indicate the strongest cell death activity in *A. suspensa* cells for *Alhid*[Ala2]. From eight test crosses between the two *Assryα-tTA* driver lines and four TRE- *Alhid*[Ala2] lethal effector lines, two hybrid strains yielded 100% lethality, with one being 96% embryonic lethal (with no survival past the first larval instar).

Similar to the RIDL female-lethality system, both the *Dmhid*[Ala5] and *Alhid*[Ala2] lethal effectors were modified by inserting the sex-specifically spliced *Cctra intron* (*CctraI*) in the 5′ region of the respective *hid* genes (Schetelig and Handler, 2012b; Ogaugwu *et al.*, 2013). The use of *TREhs43-CctraI-Dmhid*[Ala5] in medfly and *TREhs43-CctraI-Alhid*[Ala2] in caribfly with their driver lines both resulted in 100% female lethality in the absence of Tet in their respective species. For caribfly, two transgenic embryonic sexing strains (TESS) double homozygous for the driver and effector constructs exhibited total female lethality in more than 30,000 embryos tested.

Similar to *A. suspensa*, the same driver and lethal effector transgene constructs were used to create TESS strains in the mexfly, *A. ludens*. Unlike *A. suspensa,* however, the double homozygous driver/effector parental females reared on Tet-free diet were sterile, due to a cessation of vitellogenesis that resumed only after short-term feeding of the antibiotic (Schetelig *et al.*, 2016). Once fully vitellogenic, mated females removed from Tet-diet were fertile and yielded viable progeny that were up to 99% male. This result suggested that, in addition to the expected post-zygotic lethality in early embryos, a pre-zygotic cell lethality effect occurred in maternal oocytes that was not observed from the same constructs in *A. suspensa*. Potentially the *A. suspensa sry-α* promoter functions abnormally in *A. ludens,* or the specificity of *sry-α* function varies, which is consistent with similar results in *Lucilia cuprina* (Yan *et al.*, 2017).

Tet-Off transgenic strains for conditional lethality are an effective means of improving SIT in tephritid species. While both RIDL and the embryonic lethality systems are effective in tephritids, embryonic lethality is preferable for larval pests, preventing crop loss in the first generation after release (similar to classical SIT). It is also preferable for sexing by female lethality in all insects, since costs for rearing female larvae are avoided. Further advances in the development and use of transgenic strains to control the population of tephritid species are anticipated.

An important consideration for any genetic manipulation of an insect species, and especially for strains that will be mass-reared for field release, is the potential for genetic breakdown due to primary-site spontaneous mutations in the transgenes that affect or eliminate the transformant phenotype, or selection for second-site inherent suppressors or modifiers of the transformant phenotype. To test for these possibilities resulting in the breakdown of a genetically modified insect strain for improved biocontrol, a 1.2 million zygote population of the *D. melanogaster* strain for Tet-Off embryonic lethality (Horn and Wimmer, 2003) was reared to assess the frequency of heritable adult survival on a restrictive tetracycline-free diet (Zhao *et al.*, 2020). It was discovered that primary-site lethal revertant indels occurred in the F_1 generation at a frequency of 5.8×10^{-6}, in addition to twice as many maternal-effect survivors. While the primary-site survivors could be of concern due to their persistence in the environment, they would still be susceptible to control by the lethality system. Survivors that resulted from second-site modifiers, however, are likely to be resistant to the lethality system and such a resistance mechanism could be expected to introgress into the field population, as has occurred for other types of population control, especially chemical-based insecticides.

21.10 Gene-edited Strains for Improved Population Control of Tephritids

While transposon-mediated germline transformation has been the primary method for insect genetic modification and, in particular, transgenesis resulting from the ectopic genomic insertion of homologous or heterologous DNA, genetic modifications using gene editing methodologies have become more common. A primary benefit of gene editing is the ability to target mutations in a known sequence or target a sequence substitution for transgenesis. However, a current limitation to this approach is the size of the sequence that can be efficiently substituted. The first two commonly used methods for gene editing in insects, zinc-finger nucleases (ZFN) or transcriptional activator-like effector nucleases (TALENs) (Reid and O'Brochta, 2016), have been superseded more recently by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-mediated non-homologous end joining (NHEJ) and homology-directed repair (HDR) modifications. The use of these methods in insects is addressed in more detail by Ahmed and Wimmer, Chapter 5, and Concha and Papa, Chapter 7, this volume, but for tephritid species, most modifications have been limited to targeted NHEJ null-mutations for CRISPR gene editing proof-of-principle or functional verification and analysis of the targeted gene. However, actual transgenesis mediated by HDR has also been achieved in several tephritid species, resulting in temperature-dependent gene function due to amino acid substitutions and targeted introduction or mutation of marker genes.

The first gene editing by HDR in a tephritid was successfully achieved in *C. capitata* to convert the green fluorescent to a blue fluorescent protein marker in a transgenic line as proof-of-principle (Aumann *et al.,* 2018). The approach involved injecting pre-assembled CRISPR/Cas9 ribonucleoprotein complexes using different guide RNAs and a short single-stranded oligodeoxynucleotide donor into preblastodermal embryos. Eighty-six per cent fertile and individually backcrossed G_0 individuals generated 57–90% knock-in rates within their total offspring and 70–96% knock-in rates within their phenotypically mutant offspring. Such efficient approaches could also be used in other tephritids and insects to introduce mutations that do not produce a screenable phenotype, yet allow the positive identification of mutants with minimal difficulty.

Another HDR approach for potential population control, originally demonstrated in *Drosophila suzukii*, was the substitution of the 3′ coding sequence of the *transformer-2* (*tra-2*) sex-determination gene with an *in vitro* mutated sequence including a non-synonymous nucleotide resulting in an amino acid substitution resulting in a *tra-2*^{ts2} temperature-sensitive mutation, linked to a downstream DsRed marker gene (Li and Handler, 2017). At permissive temperatures of 20°C and below, XX individuals developed as normal fertile females, but at the expected non-permissive temperature of 29°C, flies did not survive metamorphosis. However, at 26°C, XX individuals did survive and exhibited several attributes of sex reversal originally observed in chemically induced *tra-2*^{ts2} mutants in *D. melanogaster* reared at 29°C (Belote and Baker, 1982), including foreleg sex-combs, male genital structures (with an ovipositor that is eliminated or truncated), abdominal posterior pigmentation and dysmorphic gonads. In addition, XX;*Dstra-2*^{ts2} individuals shifted to 29°C as pharate adults could survive, and exhibited male-specific wing spot pigmentation, and XY;*Dstra-2*^{ts2} males were sterile at 26°C. Ideally, a *Dstra-2*^{ts2} line that could survive at 29°C with XX females developing as fully differentiated, albeit sterile, males capable of mating would provide a unique strain for SIT where both XX and XY zygotes reared at the non-permissive temperature would result in sterile males. Nevertheless, rearing of the *Dstra-2*^{ts2} strain at 26°C still has the advantages of producing sterile XY male progeny, and XX sterile females that do not mate (eliminating mating competition with females in the field) and do not oviposit, resulting in crop damage. The same gene editing approach was taken with *A. suspensa* with the expectation that this tropical pest

could survive at 29°C, resulting in fully differentiated XX; *Astra-2*ts2 sterile phenotypic males; however, the required HDR integration has thus far not been observed (J. Li and A.M.H., unpublished).

In *C. capitata*, an HDR gene editing approach to induce similar SNPs was successful in the *transformer-2* gene, resulting in a 100% XX female-to-male phenotypic conversion in homozygous flies reared at 29°C for the *Cctra-2*ts2 mutation (Aumann *et al.*, 2020). However, in the resulting line, it was not possible to identify a permissive temperature for the mutation allowing the rearing of a *Cctra-2*ts2 homozygous line, as lowering the temperature below 18.5°C interfered with regular breeding of the flies.

A similar HDR gene editing approach was taken by creating a temperature-sensitive embryonic lethal mutation in the Queensland fruit fly, *Bactrocera tryoni*, with the expectation that its expression could be made female-specific for temperature-dependent genetic sexing in rearing (Choo *et al.*, 2020). Here a non-synonymous point mutation was introduced into the cognate of the *D. melanogaster shibire* (*shi*) gene that causes embryonic lethality at 29°C, which was used with CRISPR/Cas9 in *B. tryoni* to create the orthologous *shibire* temperature sensitive-1 (*shi*ts1) mutation. However, unlike *D. melanogaster*, and similar to the *Cctra2*ts2 homozygous line, *B. tryoni shi*ts1 homozygotes generally failed to survive at the permissive temperature of 21°C, limiting its use for practical application. Despite these early setbacks, the creation of temperature-sensitive conditional mutations, typically by a single nucleotide substitution, should be a highly worthwhile and straightforward application of CRISPR gene editing, to elucidate gene function and the development of conditional lethal and sterile strains for population control.

Acknowledgements

References

Andrews, B.J., Beatty, L.G. and Sadowski, P.D. (1986) Site-specific recombination of the yeast plasmid two-micron circle: intermediates in the binding process. *Basic Life Sciences* 40, 407–424.

Ant, T., Koukidou, M., Rempoulakis, P., Gong, H.F., Economopoulos, A. *et al.* (2012) Control of the olive fruit fly using genetics-enhanced sterile insect technique. *BMC Biology* 10, 51.

Aumann, R.A., Schetelig, M.F. and Häcker, I. (2018) Highly efficient genome editing by homology-directed repair using Cas9 protein in Ceratitis capitata. *Insect Biochemistry and Molecular Biology* 101, 85–93.

Aumann, R.A., Häcker, I. and Schetelig, M.F. (2020) Female-to-male sex conversion in *Ceratitis capitata* by CRISPR/Cas9 HDR-induced point mutations in the sex determination gene transformer-2. *Scientific Reports* 10, 18611.

Baer, A. and Bode, J. (2001) Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes. *Current Opinion in Biotechnology* 12, 473–480.

Bello, B., Resendez-Perez, D. and Gehring, W.J. (1998) Spatial and temporal targeting of gene expression in *Drosophila* by means of a tetracycline-dependent transactivator system. *Development* 125, 2193–2202.

Belote, J.M. and Baker, B.S. (1982) Sex determination in *Drosophila melanogaster*: analysis of transformer-2, a sex-transforming locus. *Proceedings of the National Academy of Sciences USA* 79, 1568–1572.

Benedict, M.Q., Salazar, C.E. and Collins, F.H. (1995) A new dominant selectable marker for genetic transformation: Hsp70-opd. *Insect Biochemistry and Molecular Biology* 25, 1061–1065.

Bischof, J., Maeda, R.K., Hediger, M., Karch, F. and Basler, K. (2007) An optimized transgenesis system for *Drosophila* using germ-line-specific phiC31 integrases. *Proceedings of the National Academy of Sciences USA* 104, 3312–3317.

Bonizzoni, M., Gomulski, L.M., Malacrida, A.R., Capy, P. and Gasperi, G. (2007) Highly similar *piggyBac* transposase-like sequences from various *Bactrocera* (Diptera, Tephritidae) species. *Insect Molecular Biology* 16, 645–650.

Cary, L.C., Goebel, M., Corsaro, B.G., Wang, H.G., Rosen, E. and Fraser, M.J. (1989) Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. *Virology* 172, 156–169.

Catteruccia, F., Godfray, H.C. and Crisanti, A. (2003) Impact of genetic manipulation on the fitness of *Anopheles stephensi* mosquitoes. *Science* 299, 1225–1227.

Catteruccia, F., Benton, J.P. and Crisanti, A. (2005) An *Anopheles* transgenic sexing strain for vector control. *Nature Biotechnology* 23, 1414–1417.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. (1994) Green fluorescent protein as a marker for gene expression. *Science* 263, 802–805.

Choo, A., Fung, E., Chen, I.Y., Saint, R., Crisp, P. and Baxter, S.W. (2020). Precise single base substitution in the shibire gene by CRISPR/Cas9-mediated homology directed repair in *Bactrocera tryoni*. *BMC Genetics* 21, Article 127.

Condon, K.C., Condon, G.C., Dafa'alla, T.H., Forrester, O.T., Phillips, C.E. *et al.* (2007a) Germ-line transformation of the Mexican fruit fly. *Insect Molecular Biology* 16, 573–580.

Condon, K.C., Condon, G.C., Dafa'alla, T.H., Fu, G., Phillips, C.E. *et al.* (2007b) Genetic sexing through the use of Y-linked transgenes. *Insect Biochemistry and Molecular Biology* 37, 1168–1176.

Dafa'alla, T.H., Condon, G.C., Condon, K.C., Phillips, C.E., Morrison, N.I. *et al.* (2006) Transposon-free insertions for insect genetic engineering. *Nature Biotechnology* 24, 820–821.

Davis, I., Girdham, C.H. and O'Farrell, P.H. (1995) A nuclear GFP that marks nuclei in living *Drosophila* embryos; maternal supply overcomes a delay in the appearance of zygotic fluorescence. *Developmental Biology* 170, 726–729.

Engels, W.R. (2007) Hybrid dysgenesis in *Drosophila melanogaster*: rules of inheritance of female sterility. *Genetical Research* 89, 407–424.

FAO/IAEA (2006) *Status and Risk Assessment of the Use of Transgenic Arthropods in Plant Protection*. International Atomic Energy Agency, Vienna.

Franz, G. and Savakis, C. (1991) *Minos*, a new transposable element from *Drosophila hydei*, is a member of the Tc1-like family of transposons. *Nucleic Acids Research* 19, 6646.

Franz, G., Loukeris, T.G., Dialektaki, G., Thompson, C.R. and Savakis, C. (1994) Mobile *Minos* elements from *Drosophila hydei* encode a two-exon transposase with similarity to the paired DNA-binding domain. *Proceedings of the National Academy of Sciences USA* 91, 4746–4750.

Fraser, M.J., Smith, G.E. and Summers, M.D. (1983) Acquisition of host cell DNA sequences by Baculoviruses: relationship between host DNA insertions and FP mutants of *Autographa californica* and *Galleria mellonella* nuclear polyhedrosis viruses. *Journal of Virology* 47, 287–300.

Fraser, M.J., Cary, L., Boonvisudhi, K. and Wang, H.G. (1995) Assay for movement of Lepidopteran transposon IFP2 in insect cells using a baculovirus genome as a target DNA. *Virology* 211, 397–407.

Fu, G., Condon, K.C., Epton, M.J., Gong, P., Jin, L. *et al.* (2007) Female-specific insect lethality engineered using alternative splicing. *Nature Biotechnology* 25, 353–357.

Furlong, E.E., Profitt, D. and Scott, M.P. (2001) Automated sorting of live transgenic embryos. *Nature Biotechnology* 19, 153–156.

Genc, H., Schetelig, M.F., Nirmala, X. and Handler, A.M. (2016). Germline transformation of the olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), with a *piggyBac* transposon vector. *Turkish Journal of Biology* 40, 845–855.

Gong, P., Epton, M.J., Fu, G., Scaife, S., Hiscox, A. *et al.* (2005) A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nature Biotechnology* 23, 453–456.

Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences USA* 89, 5547–5551.

Groth, A.C., Fish, M., Nusse, R. and Calos, M.P. (2004) Construction of transgenic *Drosophila* by using the site-specific integrase from phage phiC31. *Genetics* 166, 1775–1782.

Handler, A.M. (2000) An introduction to the history and methodology of insect gene transfer. In: Handler, A.M. and James, A.A. (eds) *Insect Transgenesis: Methods and Applications*. CRC Press, Boca Raton, Florida, pp. 3–26.

Handler, A.M. (2002a) Prospects for using genetic transformation for improved SIT and new biocontrol methods. *Genetica* 116, 137–149.

Handler, A.M. (2002b) Use of the *piggyBac* transposon for germ-line transformation of insects. *Insect Biochemistry and Molecular Biology* 32, 1211–1220.

Handler, A.M. (2003) Isolation and analysis of a new *hopper* hAT transposon from the *Bactrocera dorsalis* white eye strain. *Genetica* 118, 17–24.

Handler, A.M. (2004) Understanding and improving transgene stability and expression in insects for SIT and conditional lethal release programs. *Insect Biochemistry and Molecular Biology* 34, 121–130.

Handler, A.M. and Gomez, S.P. (1996) The *hobo* transposable element excises and has related elements in tephritid species. *Genetics* 143, 1339–1347.

Handler, A.M. and Gomez, S.P. (1997) A new *hobo*, *Ac*, *Tam3* transposable element, *hopper*, from *Bactrocera dorsalis* is distantly related to *hobo* and *Ac*. *Gene* 185, 133–135.

Handler, A.M. and Harrell, R.A. (1999) Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector. *Insect Molecular Biology* 8, 449–457.

Handler, A.M. and Harrell, R.A. (2001a) Polyubiquitin-regulated DsRed marker for transgenic insects. *Biotechniques* 31, 820, 824–828.

Handler, A.M. and Harrell, R.A. (2001b) Transformation of the Caribbean fruit fly, *Anastrepha suspensa*, with a *piggyBac* vector marked with polyubiquitin-regulated GFP. *Insect Biochemistry and Molecular Biology* 31, 199–205.

Handler, A.M. and McCombs, S.D. (2000) The *piggyBac* transposon mediates germ-line transformation in the Oriental fruit fly and closely related elements exist in its genome. *Insect Molecular Biology* 9, 605–612.

Handler, A.M. and O'Brochta, D.A. (2012) Transposable elements for insect transformation. In: Gilbert, L.I. (ed.) *Insect Biochemistry and Molecular Biology*. Academic Press, London, pp. 90–133.

Handler, A.M. and Schetelig, M.F. (2020) The hAT-family transposable element, *hopper*, from *Bactrocera dorsalis* is a functional vector for insect germline transformation. *BMC Genetics* 21, 137.

Handler, A.M., McCombs, S.D., Fraser, M.J. and Saul, S.H. (1998) The lepidopteran transposon vector, *piggyBac*, mediates germ-line transformation in the Mediterranean fruit fly. *Proceedings of the National Academy of Sciences USA* 95, 7520–7525.

Handler, A.M., Zimowska, G.J. and Horn, C. (2004) Post-integration stabilization of a transposon vector by terminal sequence deletion in *Drosophila melanogaster*. *Nature Biotechnology* 22, 1150–1154.

Handler, A.M., Zimowska, G.J. and Armstrong, K.F. (2008) Highly similar *piggyBac* elements in *Bactrocera* that share a common lineage with elements in noctuid moths. *Insect Molecular Biology* 17, 387–393.

Heinrich, J.C. and Scott, M.J. (2000) A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. *Proceedings of the National Academy of Sciences USA* 97, 8229–8232.

Horn, C. and Handler, A.M. (2005) Site-specific genomic targeting in *Drosophila*. *Proceedings of the National Academy of Sciences USA* 102, 12483–12488.

Horn, C. and Wimmer, E.A. (2003) A transgene-based, embryo-specific lethality system for insect pest management. *Nature Biotechnology* 21, 64–70.

Horn, C., Schmid, B.G., Pogoda, F.S. and Wimmer, E.A. (2002) Fluorescent transformation markers for insect transgenesis. *Insect Biochemistry and Molecular Biology* 32, 1221–1235.

Hoy, M.A. (2003) Transgenic insects for pest management programs: status and prospects. *Environmental Biosafety Research* 2, 61–64.

Irvin, N., Hoddle, M.S., O'Brochta, D.A., Carey, B. and Atkinson, P.W. (2004) Assessing fitness costs for transgenic *Aedes aegypti* expressing the GFP marker and transposase genes. *Proceedings of the National Academy of Sciences USA* 101, 891–896.

Koukidou, M., Klinakis, A., Reboulakis, C., Zagoraiou, L., Tavernarakis, N. *et al.* (2006) Germ line transformation of the olive fly *Bactrocera oleae* using a versatile transgenesis marker. *Insect Molecular Biology* 15, 95–103.

Labbé, G.M., Nimmo, D.D. and Alphey, L. (2010) *piggybac*- and PhiC31-mediated genetic transformation of the Asian tiger mosquito, *Aedes albopictus* (Skuse). *PLoS Neglected Tropical Diseases* 4, e788.

Lee, H.S., Simon, J.A. and Lis, J.T. (1988) Structure and expression of ubiquitin genes of *Drosophila melanogaster*. *Molecular and Cellular Biology* 8, 4727–4735.

Li, J.W. and Handler, A.M. (2017) Temperature-dependent sex-reversal by a transformer-2 gene-edited mutation in the spotted wing drosophila, *Drosophila suzukii*. *Scientific Reports* 7, 12363.

Loukeris, T.G., Arca, B., Livadaras, I., Dialektaki, G. and Savakis, C. (1995a) Introduction of the transposable element *Minos* into the germ line of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences USA* 92, 9485–9489.

Loukeris, T.G., Livadaras, I., Arca, B., Zabalou, S. and Savakis, C. (1995b) Gene transfer into the medfly, *Ceratitis capitata*, with a *Drosophila hydei* transposable element. *Science* 270, 2002–2005.

Marois, E., Scali, C., Soichot, J., Kappler, C., Levashina, E.A. and Catteruccia, F. (2012) High-throughput sorting of mosquito larvae for laboratory studies and for future vector control interventions. *Malaria Journal* 11, 302.

Marubbi, T., Cassidy, C., Miller, E., Koukidou, M., Martin-Rendon, E. *et al.* (2017) Exposure to genetically engineered olive fly (*Bactrocera oleae*) has no negative impact on three non-target organisms. *Scientific Reports* 7, 11478.

McCombs, S.D. and Saul, S.H. (1992) Linkage analysis of five new genetic markers of the oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae). *Journal of Heredity* 83, 199–203.

McInnis, D.O. (1993) Size differences between normal and irradiated sperm heads in mated female Mediterranean fruit flies (Diptera: Tephritidae). *Annals of the Entomological Society of America* 86, 305–308.

Meza, J.S., Nirmala, X., Zimowska, G.J., Zepeda-Cisneros, C.S. and Handler, A.M. (2011) Development of transgenic strains for the biological control of the Mexican fruit fly, *Anastrepha ludens*. *Genetica* 139, 53–62.

Meza, J.S., Diaz-Fleischer, F., Sanchez-Velasquez, L.R., Zepeda-Cisneros, C.S., Handler, A.M. and Schetelig, M.F. (2014a) Fitness cost implications of PhiC31-mediated site-specific integrations in target-site strains of the Mexican fruit fly, *Anastrepha ludens* (Diptera: Tephritidae). *PLoS ONE* 9, e109690.

Meza, J.S., Schetelig, M.F., Zepeda-Cisneros, C.S. and Handler, A.M. (2014b) Male-specific Y-linked transgene markers to enhance biologically-based control of the Mexican fruit fly, *Anastrepha ludens* (Diptera: Tephritidae). *BMC Genetics* 15 (Suppl. 2), S4.

Michel, K., Stamenova, A., Pinkerton, A.C., Franz, G., Robinson, A.S. *et al.* (2001) Hermes-mediated germ-line transformation of the Mediterranean fruit fly *Ceratitis capitata*. *Insect Molecular Biology* 10, 155–162.

Mitchell, W.C. and Saul, S.H. (1990) Current control methods for the Mediterranean fruit fly, *Ceratitis capitata*, and their application in the USA. *Review of Agricultural Entomology* 78, 923–940.

Morrison, N.I., Franz, G., Koukidou, M., Miller, T.A., Saccone, G. *et al.* (2010) Genetic improvements to the Sterile Insect Technique for agricultural pests. *Asia-Pacific Journal of Molecular Biology and Biotechnology* 18, 275–295.

Nimmo, D.D., Alphey, L., Meredith, J.M. and Eggleston, P. (2006) High efficiency site-specific genetic engineering of the mosquito genome. *Insect Molecular Biology* 15, 129–136.

Nirmala, X., Zimowska, G.J. and Handler, A.M. (2009) Characterization of the proteasome β2 subunit gene and its mutant allele in the tephritid fruit fly pest, *Anastrepha suspensa*. *Insect Molecular Biology* 18, 333–340.

Nirmala, X., Olson, S.R., Holler, T.C., Cho, K.H. and Handler, A.M. (2011) A DsRed fluorescent protein marker under polyubiquitin promoter regulation allows visual and amplified gene detection of transgenic Caribbean fruit flies in field traps. *BioControl* 56, 333–340.

Oberstein, A., Pare, A., Kaplan, L. and Small, S. (2005) Site-specific transgenesis by Cre-mediated recombination in Drosophila. *Nature Methods* 2, 583–585.

Ogaugwu, C.E., Schetelig, M.F. and Wimmer, E.A. (2013) Transgenic sexing system for *Ceratitis capitata* (Diptera: Tephritidae) based on female-specific embryonic lethality. *Insect Biochemistry and Molecular Biology* 43, 1–8.

Orozco-Davila, D., Hernandez, R., Meza, S. and Dominguez, J. (2007) Sexual competitiveness and compatibility between mass-reared sterile flies and wild populations of *Anastrepha ludens* (Diptera: Tephritidae) from different regions in Mexico. *Florida Entomologist* 90, 19–26.

Pane, A., Salvemini, M., Delli, B.P., Polito, C. and Saccone, G. (2002) The transformer gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. *Development* 129, 3715–3725.

Raphael, K.A., Whyard, S., Shearman, D., An, X. and Frommer, M. (2004) *Bactrocera tryoni* and closely related pest tephritids – molecular analysis and prospects for transgenic control strategies. *Insect Biochemistry and Molecular Biology* 34, 167–176.

Raphael, K.A., Shearman, D.C., Streamer, K., Morrow, J.L., Handler, A.M. and Frommer, M. (2011) Germline transformation of the Queensland fruit fly, *Bactrocera tryoni*, using a *piggyBac* vector in the presence of endogenous piggyBac elements. *Genetica* 139, 91–97.

Reid, W. and O'Brochta, D.A. (2016) Applications of genome editing in insects. *Current Opinion in Insect Science* 13, 43–54.

Robinson, A.S. and Franz, G. (2000) The application of transgenic insect technology in the sterile insect technique. In: Handler, A.M. and James, A.A. (eds) *Insect Transgenesis: Methods and Applications*. CRC Press, Boca Raton, Florida, pp. 307–319.

Rossier, Y. and Rosenthal, H. (1992) Genetics of the Mediterranean fruit fly (Diptera: Tephritidae) morphological mutants on chromosome five. *Annals of the Entomological Society of America* 85(4), 525–531.

Rubin, G.M. and Spradling, A.C. (1982) Genetic transformation of *Drosophila* with transposable element vectors. *Science* 218, 348–353.

Sarkar, A., Atapattu, A., Belikoff, E.J., Heinrich, J.C., Li, X. *et al.* (2006) Insulated *piggyBac* vectors for insect transgenesis. *BMC Biotechnology* 6, 27.

Schetelig, M.F. and Handler, A.M. (2012a) Strategy for enhanced transgenic strain development for embryonic conditional lethality in *Anastrepha suspensa*. *Proceedings of the National Academy of Sciences USA* 109, 9348–9353.

Schetelig, M.F. and Handler, A.M. (2012b) A transgenic embryonic sexing system for *Anastrepha suspensa* (Diptera: Tephritidae). *Insect Biochemistry and Molecular Biology* 42, 790–795.

Schetelig, M.F. and Handler, A.M. (2013a) A functional comparison of the 3xP3 promoter by recombinase-mediated cassette exchange in *Drosophila* and a tephritid fly, *Anastrepha suspensa*. *G3: Genes Genomes Genetics* 3, 687–693.

Schetelig, M.F. and Handler, A.M. (2013b) Y-linked markers for improved population control of the tephritid fruit fly pest, *Anastrepha suspensa*. In: Vilcinskas, A. (ed.) *Insect Biotechnology*. Springer, Dordrecht, Netherlands, pp. 169–194.

Schetelig, M.F., Horn, C., Handler, A.M. and Wimmer, E.A. (2007) Development of an embryonic lethality system for SIT in *Ceratitis capitata*. In: Vreysen, M.J.B, Robinson, A.S. and Hendrichs, J. (eds) *Area-wide Control of Insect Pests: from research to field implementation*. Springer, Dordrecht, Netherlands, pp. 85–93.

Schetelig, M.F., Scolari, F., Handler, A.M., Gasperi, G. and Wimmer, E.A. (2008) New genetic tools for improving SIT in *Ceratitis capitata*: embryonic lethality and sperm marking. In: Sugayama, R., Zucchi, R., Ovruski, S. and Sivinski, J. (eds) *Fruit Flies of Economic Importance: From Basic to Applied Knowledge*. Proceedings of 7th International Symposium on Fruit Flies of Economic Importance, Salvador, Brazil, 10–15 September 2006. Salvador, SBPC, pp. 299–305.

Schetelig, M.F., Caceres, C., Zacharopoulou, A., Franz, G. and Wimmer, E.A. (2009a) Conditional embryonic lethality to improve the sterile insect technique in *Ceratitis capitata* (Diptera: Tephritidae). *BMC Biology* 7, 4.

Schetelig, M.F., Scolari, F., Handler, A.M., Kittelmann, S., Gasperi, G. and Wimmer, E.A. (2009b) Site-specific recombination for the modification of transgenic strains of the Mediterranean fruit fly *Ceratitis capitata*. *Proceedings of the National Academy of Sciences USA* 106, 18171–18176.

Schetelig, M.F., Götschel, F., Viktorinova, I., Handler, A.M. and Wimmer, E.A. (2011a) Recombination technologies for enhanced transgene stability in bioengineered insects. *Genetica* 139, 71–78.

Schetelig, M.F., Nirmala, X. and Handler, A.M. (2011b) Pro-apoptotic cell death genes, *hid* and *reaper*, from the tephritid pest species, *Anastrepha suspensa*. *Apoptosis* 16, 759–768.

Schetelig, M.F., Targovska, A., Meza, J.S., Bourtzis, K. and Handler, A.M. (2016) Tetracycline-suppressible female lethality and sterility in the Mexican fruit fly, *Anastrepha ludens*. *Insect Molecular Biology* 25, 500–508.

Schetelig, M.F., Yan, Y., Zhao, Y. and Handler, A.M. (2019) Genomic targeting by recombinase-mediated cassette exchange in the spotted wing drosophila, *Drosophila suzukii*. *Insect Molecular Biology* 28, 187–195.

Schotta, G., Ebert, A., Dorn, R. and Reuter, G. (2003) Position-effect variegation and the genetic dissection of chromatin regulation in *Drosophila*. *Seminars in Cell & Developmental Biology* 14, 67–75.

Scolari, F., Schetelig, M.F., Bertin, S., Malacrida, A.R., Gasperi, G. and Wimmer, E.A. (2008) Fluorescent sperm marking to improve the fight against the pest insect *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae). *New Biotechnology* 25, 76–84.

Siegal, M.L. and Hartl, D.L. (1996) Transgene coplacement and high efficiency site-specific recombination with the Cre/loxP system in *Drosophila*. *Genetics* 144, 715–726.

Smith, R.C., Walter, M.F., Hice, R.H., O'Brochta, D.A. and Atkinson, P.W. (2007) Testis-specific expression of the *β*2 tubulin promoter of *Aedes aegypti* and its application as a genetic sex-separation marker. *Insect Molecular Biology* 16, 61–71.

Smyth, K.A. and Belote, J.M. (1999) The dominant temperature-sensitive lethal DTS7 of *Drosophila melanogaster* encodes an altered 20S proteasome beta-type subunit. *Genetics* 151, 211–220.

Steller, H. and Pirrotta, V. (1985) A transposable P vector that confers selectable G418 resistance to *Drosophila* larvae. *EMBO Journal* 4, 167–171.

Sundararajan, P., Atkinson, P.W. and O'Brochta, D.A. (1999) Transposable element interactions in insects: crossmobilization of *hobo* and *Hermes*. *Insect Molecular Biology* 8, 359–368.

Thibault, S.T., Singer, M.A., Miyazaki, W.Y., Milash, B., Dompe, N.A., *et al.* (2004) A complementary transposon tool kit for *Drosophila melanogaster* using *P* and *piggyBac*. *Nature Genetics* 36, 283–287.

Thomas, D.D., Donnelly, C.A., Wood, R.J. and Alphey, L.S. (2000) Insect population control using a dominant, repressible, lethal genetic system. *Science* 287, 2474–2476.

Vella, M., Gould, F. and Lloyd, A. (2021) Mathematical modeling of genetic pest management through female-specific lethality: is one locus better than two? *Evolutionary Applications* 10.1111/eva.13228.

Venken, K.J. and Bellen, H.J. (2012) Genome-wide manipulations of *Drosophila melanogaster* with transposons, Flp recombinase, and PhiC31 integrase. *Methods in Molecular Biology* 859, 203–228.

Venken, K.J., He, Y., Hoskins, R.A. and Bellen, H.J. (2006) P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in *D. melanogaster*. *Science* 314, 1747–1751.

Warren, W.D., Atkinson, P.W. and O'Brochta, D.A. (1994) The *Hermes* transposable element from the house fly, *Musca domestica*, is a short inverted repeat-type element of the *hobo*, *Ac*, and *Tam3* (hAT) element family. *Genetical Research* 64, 87–97.

Yan, Y., Linger, R. J. and Scott, M. J. (2017) Building early-larval sexing systems for genetic control of the Australian sheep blow fly *Lucilia cuprina* using two constitutive promoters. *Scientific Reports* 7, 2538.

Young, O.P., Ingebritsen, S.P. and Foudin, A.S. (2000) Regulation of transgenic arthropods and other invertebrates in the United States. In: Handler, A.M. and James, A.A. (eds) *Insect Transgenesis: Methods and Applications*. CRC Press, Boca Raton, Florida, pp. 369–371.

Zhao, Y., Schetelig, M.F., and Handler, A.M. (2020) Genetic breakdown of a Tet-off conditional lethality system for insect population control. *Nature Communications* 11, 3095.

Zimowska, G.J., Nirmala, X. and Handler, A.M. (2009) The beta2-tubulin gene from three tephritid fruit fly species and use of its promoter for sperm marking. *Insect Biochemistry and Molecular Biology* 39, 508–515.

Zwiebel, L.J., Saccone, G., Zacharopoulou, A., Besansky, N.J., Favia, G. *et al.* (1995) The white gene of *Ceratitis capitata*: a phenotypic marker for germline transformation. *Science* 270, 2005–2008.

22   Antiviral Effectors for Mosquito Transgenesis

Alexander W.E. Franz*

*Department of Veterinary Pathobiology, College of Veterinary Medicine,
University of Missouri, Columbia, MO 65211, USA*

22.1   Introduction

Mosquito-transmitted arthropod-borne viruses ('arboviruses') such as the dengue viruses (DENV 1-4), chikungunya virus (CHIKV), Zika virus (ZIKV), Japanese encephalitis virus (JEV) and yellow fever virus (YFV) are important pathogens predominantly affecting human populations in the tropical regions of the world (Weaver and Reisen, 2010; Liu-Helmersson *et al.*, 2019; Mordecai *et al.*, 2020). The four serotypes of DENV, arguably the most important mosquito-borne arboviruses affecting humans, infect 100–390 million people annually and place 2 billion people in 100 countries at risk of contracting dengue disease due to mosquito transmission (Guzman *et al.*, 2010; Simmons *et al.*, 2012; Bhatt *et al.*, 2013; Roth *et al.*, 2014). Within two decades, three mosquito-borne viruses of Old World origin – West Nile virus (WNV) in 1999, CHIKV in 2013 and ZIKV in 2015 – entered the Western Hemisphere and caused epidemic outbreaks in the Americas and in the Caribbean Islands (Nash *et al.*, 2001; Blitvich, 2008; Weaver, 2014; Chang *et al.*, 2016; Pastula *et al.*, 2016; Carrera *et al.*, 2017). It is likely that more arboviruses will (re-)emerge over time and

eventually spread to novel regions via human trade and traffic (Gubler, 2002). Along with the viruses they transmit, mosquito vectors are also constantly increasing their global range as a result of human activity and climate change (Adams and Kapan, 2009; Gould *et al.*, 2017; Schrama *et al.*, 2020). For example, the Asian tiger mosquito (*Aedes albopictus*), originating from South-East Asia, was discovered in 1979 for the first time on the European continent in Albania and in 1985 in the USA (Texas) (Lambrechts *et al.*, 2010). Meanwhile, the mosquito species has established itself in 40 US states (Hahn *et al.*, 2017).

Many mosquito-borne arboviruses that affect global health, including those mentioned above, are flaviviruses (family: Flaviviridae; genus: *Flavivirus*) or alphaviruses (family: Togaviridae; genus: *Alphavirus*). Viruses of these two families have 11–12 kilobases (kb) positive-sense RNA genomes and their virions contain a lipid envelope (Jose *et al.*, 2009; Barrows *et al.*, 2018). Alphaviruses and most of the flaviviruses are transmitted by mosquitoes of the genera *Culex* and *Aedes* (Weaver and Barrett, 2004; Guzmán *et al.*, 2020). Typically, *Culex* spp. are vectors of those alpha- and flaviviruses, which generate neurotrophic disease manifestations in infected

---

*Email: franza@missouri.edu

vertebrates and have birds as their major animal reservoir. Most of the non-neurotropic alpha- and flaviviruses, including CHIKV, DENV1-4, ZIKV and YFV, are transmitted by *Aedes* spp. and involve primates in sylvatic transmission cycles.

The principal vectors of CHIKV, DENV1-4 and ZIKV in urban disease cycles are *Aedes aegypti* and *Ae. albopictus* (Sukhralia *et al.*, 2019). *Ae. aegypti* is a peridomestic/anthropophilic, day-biting vector having a median flight range of usually less than 100 m, with most of its daily activities occurring indoors (Harrington *et al.*, 2005; Ooi *et al.*, 2006), whereas *Ae. albopictus* has a broad vertebrate host range, including humans (Kek *et al.*, 2014). *Ae. aegypti* and *Ae. albopictus* females take multiple bloodmeals during their lifetime, increasing the risk of arbovirus transmission (Scott and Takken, 2012). Mosquitoes acquire an arbovirus through a viraemic bloodmeal taken from a vertebrate host (Franz *et al.*, 2015). The bloodmeal enters the lumen of the mosquito midgut, where it is digested. Virions within the bloodmeal need to infect the mosquito's epithelium lining the midgut before the formation of the peritrophic matrix. Infection of the midgut epithelial cells occurs via receptor-mediated endocytosis. Following a replication cycle in the midgut epithelial cells, *de novo* synthesized virions are ready to exit the midgut tissue and disseminate to secondary tissues, including neuro tissue, fat body, ovaries and the salivary glands, thereby persistently and systemically infecting the mosquito. Once the salivary glands are infected, the virus can be released along with saliva whenever the female mosquito is probing on a vertebrate.

In common situations in which an arbovirus is well adapted to its mosquito vector, infection with the virus does not cause any obvious pathology to the mosquito (Clem, 2016). Furthermore, not every mosquito species or strain is a competent vector for every arbovirus species or strain, indicating that there is a highly specific molecular interaction between mosquito vector and virus (Rückert and Ebel, 2018). Receptor recognition at the midgut epithelium surface is a major bottleneck for bloodmeal-acquired arboviruses. Once inside the cell, arboviruses

are confronted with several antiviral immune responses, including RNA interference, which the viruses need to overcome in order to establish an infection (Sanchez-Vargas *et al.*, 2004, 2009; Angleró-Rodríguez *et al.*, 2017). Another hurdle is the exit from the midgut and successful infection of secondary tissues (Franz *et al.*, 2015; Dong *et al.*, 2017; Kantor *et al.*, 2018; Cui *et al.*, 2019). The efficiency of arbovirus dissemination from the midgut can vary depending on the mosquito-to-strain–virus-strain combination. Once revealed at the molecular level, certain essential interactions between virus and cellular components could present suitable targets for antiviral effectors that would be able to block or modulate these interactions, thereby inhibiting replication or systemic infection of the virus.

In most endemic regions of the world, current arbovirus control strategies rely on vector control efforts, including the use of (insecticide-treated) bednets and window curtains, removal of mosquito oviposition sites around premises and residual insecticide applications (Bowman *et al.*, 2018; Herrera-Bojórquez *et al.*, 2020). The latter approach is becoming more and more problematic as mosquito populations increasingly develop resistance to many insecticides (Carlvalho and Moreira, 2017; Pereira Cabral *et al.*, 2019). Since the 1930s, highly effective, licensed vaccines have been available against YFV and JEV (Theiler and Smith, 1937; Hedge and Gore, 2017). Previously, a recombinant tetravalent DENV vaccine, Dengvaxia, had been made available to the public (Halstead and Aguiar, 2016). However, due to a controversy regarding the vaccine's efficacy and safety, it is now recommended only for those people who previously have been infected with DENV.

Other, novel concepts to control arbovirus transmission by mosquitoes in the field involve the genetic manipulation of mosquito vectors (James, 2005) (see Bottino-Rojas and James, Chapter 11; Guido *et al.*, Chapter 16, this volume). One such strategy based on population suppression uses mosquitoes that have been genetically modified to render them unable to produce viable offspring (Black *et al.*, 2011). This strategy is thoroughly

described by Arien *et al.*, Chapter 10 and Morrison, Chapter 23, this volume, and will not be further discussed here. The other concept, population replacement, uses laboratory-engineered mosquitoes, which have been genetically modified to be virus resistant and would be released in a target area where mosquitoes of the same species are transmitting a particular arbovirus (James, 2005). Considering the virus resistance phenotype to be a dominant trait, crossbreeding between laboratory-engineered and wild-type mosquitoes would eventually convert the virus-susceptible population into one that is virus resistant. In combination with a gene drive system, the required core component, when following this strategy, would be a robust antiviral effector that is expressed in a relevant tissue of the mosquito to suppress the targeted virus at an early stage during its replication cycle in the vector. In this chapter, we present and discuss the various synthetic antiviral effectors that have been designed and tested in *Ae. aegypti* to suppress infections with DENV1-4, ZIKV and CHIKV.

22.2 The Principle of *Ae. aegypti* Germline Transformation

Genetic manipulation of the *Ae. aegypti* genome is a well-established procedure and has been performed for more than two decades with three major goals in mind: (i) imposing genetic loads in the context of population reduction strategies; (ii) manipulating/reducing vector competence for arboviruses; and (iii) pursuing specific studies on gene function in the mosquito.

Ae. aegypti is an arthropod that can be relatively easily manipulated and maintained in the laboratory and has less stringent requirements for larval food composition and light conditions than other mosquito species (Jasinskiene *et al.*, 1998). Another advantage is that *Ae. aegypti* eggs can be desiccated and stored for up to 3–4 months before hatching. The following genetic components are typically used to overexpress a heterologous gene or a synthetic antiviral effector in *Ae. aegypti* (Franz *et al.,* 2006): (i) a

non-autonomous (class II DNA) transposable element (TE) as a one-time cut-and-paste transgene insertion vector; (ii) a reliable selectable marker such as a fluorescent protein under control of the synthetic, eye tissue-specific 3xP3 promoter; and (iii) a tissue-specific promoter to overexpress the antiviral effector gene. The eye marker and the effector gene cassettes are inserted between the left and right short inverted terminal repeat arms of the TE, whereas the TE's original, functional transposase coding sequence has been transferred to a separate, so-called helper plasmid and placed under control of a heat shock promoter (*hsp83*) (see O'Brochta, Chapter 1, this volume). The modified TE plasmid ('donor') and the helper plasmid are then co-injected at defined concentrations into the posterior end of preblastoderm embryos using a micromanipulator, which is connected to an air compressor. Injected eggs are incubated for 4–5 days under moist condition at 28°C and 80% relative humidity, hatched, and reared to adults. The generation 0 (G_0) adults are mated to the recipient (non-transgenic) laboratory strain. Eggs are hatched 1 week later and G_1 larvae are screened for eye tissue-specific marker expression. G_2/G_3 mosquitoes can then be tested for their transgenic phenotype based on the effector gene. *Mariner Mos1*, originating from *Drosophila mauritiana*, and *piggyBac*, originating from the lepidopteran, *Autographa californica*, are the two class II TEs that are routinely used for germline transformation of *Ae. aegypti* (Medhora *et al.*, 1988; Lobo *et al.*, 1999) (see O'Brochta, Chapter 1, this volume). Both TEs require short genomic sequence recognition motifs such as 'TA' for *mariner Mos1* and 'TTAA' for *piggyBac*, which allow their quasi-random integration into the mosquito genome (O'Brochta and Atkinson, 1996).

22.2.1 Promoters for tissue-specific effector gene expression in *Ae. aegypti*

Precise spatial and temporal antiviral effector gene expression (see Nolan and Hammond, Chapter 3, this volume) in the mosquito is critical to successfully antagonize an arbovirus,

which has been acquired by the mosquito along with a bloodmeal. Importantly, the antiviral effector needs to be expressed in tissues that are relevant for virus infection. Ideally, the antiviral effector needs to be already expressed and established in the relevant tissue before the virus is entering that tissue. The initial mosquito tissue infected by a virus such as DENV1-4 or CHIKV is the posterior midgut epithelium (Franz *et al*., 2015). Overexpression of an antiviral effector in the midgut epithelium during bloodmeal digestion would target the virus at an early stage of its infection cycle, ideally before the virus has been able to establish any infection foci in the tissue. The female midgut-specific *carboxypeptidase A* (*CPA*) promoter is controlling gene expression in the midgut epithelium of blood-fed females between 4 h and 32 h post-bloodmeal (pbm), an ideal time window to target bloodmeal-acquired arboviruses at the onset of infection (Edwards *et al*., 2000; Franz *et al*., 2006). The bloodmeal-inducible *vitellogenin 1a* (*Vg*) promoter controls gene expression in the fat body up to about 24 h pbm (Kokoza *et al*., 2000). Two constitutive promoters have been used so far for the expression of antiviral effector genes in *Ae. aegypti*: the salivary gland-specific *30 K* promoter (Mathur *et al*., 2010) and the poly-ubiquitin (*PUb*) promoter (Anderson *et al*., 2010), which is active in most mosquito tissues, including the midgut.

22.3   Synthetic Antiviral Effectors that Target and Degrade Viral RNA Genomes

22.3.1   RNA interference – the siRNA pathway in mosquitoes

Mosquitoes, like other Diptera, possess a molecular pathway termed RNA interference (RNAi) regulating the expression of endogenous and exogenous RNAs (Sanchez-Vargas *et al*., 2004) (see De Schutter and Smagghe, Chapter 4, this volume). The RNAi pathway can be further subdivided into three different branches: (i) the small interfering (si) RNA; (ii) the P-element-induced wimpy testis-associated (pi)RNA; and (iii) the micro (mi)

RNA pathways (Campbell *et al*., 2008). While the miRNA pathway predominantly regulates the expression of endogenous messenger (m)RNAs, the siRNA and piRNA pathways form an RNA-based immune system controlling the expression and replication of non-endogenous RNAs, which are of viral origin. The major function of the piRNA pathway is to restrict TE activity in the germline.

The siRNA pathway is the principal pathway antagonizing RNA virus infection and replication in somatic tissues of dipterans (Olson and Blair, 2015). The RNAse III-like enzyme, Dicer2, is a specific component of this pathway. Dicer2 senses the presence of long double-stranded (ds)RNA molecule structures in the cell cytoplasm that arise from secondary RNA structures of viral RNA genomes and/or during viral RNA replication and processes long dsRNA molecules into characteristic 21 bp RNA duplexes, which have a 2 nucleotide (nt) overhang at their 3′-OH ends (Fig. 22.1). With the help of the RNA-binding protein, R2D2, each 21 bp duplex is loaded into an siRNA-induced silencing complex (siRISC) consisting of a protein complex including Argonaute2 (Ago2), which possesses RNAse H-like endonuclease activity (Pratt and MacRae, 2009). Ago2 then unwinds the 21 bp RNA duplex. While the passenger strain is discarded, the retained 21 nt guide strain then directs siRISC to (single-stranded) RNA molecules exhibiting sequence complementarity. Upon binding, Ago2 then cleaves the RNA molecule, resulting in the destruction of viral RNAs in a homology-dependent manner (Olson and Blair, 2015).

Under natural conditions, the mosquito's siRNA pathway is not clearing arbovirus infections from mosquitoes. This might be due to measures employed by the viruses to evade or counter the RNAi response (O'Neal *et al*., 2014). Regardless, during a typical arbovirus infection, the mosquito's siRNA pathway response leads to the cleavage of viral genomes, thereby keeping the viral titre under a threshold level above which it may have detrimental consequences for the mosquito (Sanchez-Vargas *et al*., 2009; Khoo *et al*., 2010, 2013). It has been demonstrated that siRNA pathway suppression during
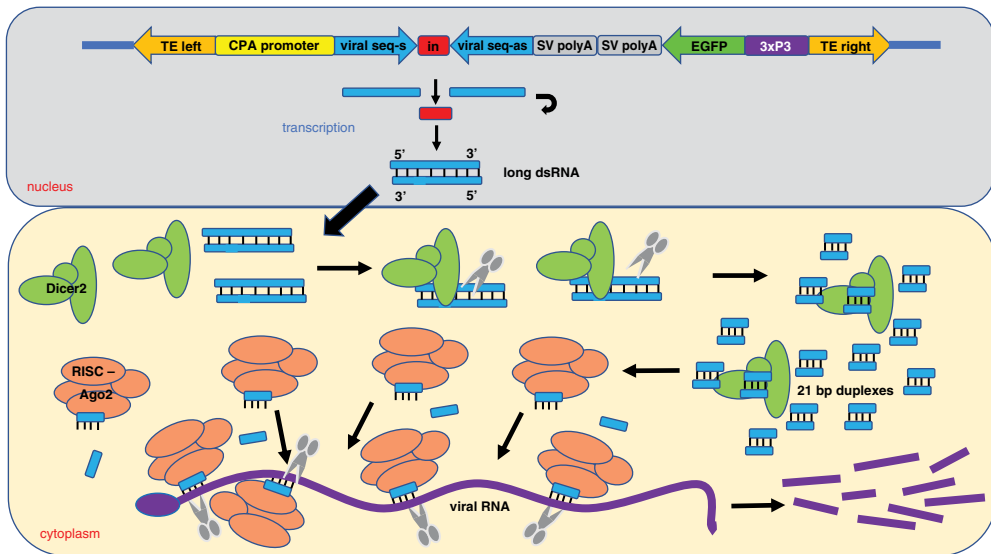
Fig. 22.1. Schematic representation of a transgene containing an inverted-repeat effector to trigger antiviral RNAi, and processing of the expressed long dsRNA by the endogenous siRNA pathway of *Aedes aegypti*. A transposable element (TE) such as *mariner Mos1* is used as a transgene insertion vector for the germline transformation of *Ae. aegypti*. Following acquisition of a bloodmeal, the IR effector is transcribed in the nucleus of midgut epithelial cells and processed into a long dsRNA molecule. The long dsRNA is exported into the cytoplasm and sensed by Dicer2, which cleaves the long dsRNA into 21 bp duplexes. Activity of the RNA-binding protein R2D2 causes the 21 bp duplexes to unwind. One of the strands (guide strand) is incorporated into the RNA-induced silencing complex (RISC), whereas the other strand (passenger strand) is discarded. Argonaute2 (Ago2) of RISC guides the complex to RNA molecules exhibiting sequence homology to the guide strand (i.e., the viral RNA genome of a virus) and cleaves the viral RNA. Abbreviations: *CPA, (Ae. aegypti) carboxypeptidase A promoter*; viral seq-s, viral seq-as, ~500 bp cDNA derived from a viral RNA genome in sense and antisense orientations, respectively; in, minor (62 nt) intron of *Ae. aegypti sialokinin I*; svA, polyadenylation signal of Simian virus 40 VP1 gene; EGFP, enhanced green fluorescent protein; 3xP3, synthetic eye tissue specific promoter; TE left, TE right, left and right inverted terminal repeats of a class II DNA transposon.

infection with a recombinant Sindbis virus (Togaviridae; *Alphavirus*) dramatically increased the virus titre in the mosquito, eventually killing the insect (Myles *et al.*, 2008; Cirimotich *et al.*, 2009). Furthermore, our extensive studies (as further described below) revealed that the mosquito's siRNA pathway can be effectively reprogrammed and utilized for antiviral effector design.

22.3.2 Long arbovirus-derived dsRNAs as triggers for the antiviral siRNA pathway in *Ae. aegypti*

The initial antiviral effector gene which had been overexpressed in transgenic *Ae. aegypti*

was a dsRNA of about 580 bp derived from the prM-M encoding region of the DENV2 genome (Franz *et al.*, 2006). The dsRNA resulted from the transcription of an inverted-repeat (IR) cDNA containing the prM-M encoding fragments in sense and antisense orientations, separated by the small intron of the *Sialokinin 1* gene (Beerntsen *et al.*, 1999) (Fig. 22.1). Expression of the IR cDNA was under control of the *CPA* promoter and a transcription terminator derived from Simian virus 40 was inserted downstream of the IR molecule. This effector gene cassette was inserted into the *mariner Mos1* TE, also containing a fluorescent eye marker expression cassette. In an initial germline transformation experiment, in which the modified TE

was used to generate DENV2-resistant *Ae. aegypti*, one line, designated Carb77, stably expressed the IR effector RNA in midguts of blood-fed females between 27 and 48 h pbm (Franz *et al.*, 2006). Inside the cells of the midgut epithelium, the transcribed dsRNA molecule of the effector was recognized and processed by the endogenous RNAi machinery, resulting in the formation of 21 bp siRNAs with sequence homology to the prM-M encoding region of the DENV2 RNA. As a consequence, Carb77 mosquitoes were highly resistant to the virus. Following oral challenge with a DENV2-containing bloodmeal, over 90% of the mosquitoes had zero virus infection in their midguts or in other tissues and did not release any virus in their saliva. However, when bypassing the midgut via intrathoracic injection of the virus, mosquitoes became strongly infected and released virus along with saliva. Thus, overexpression of the transgene established a robust midgut infection barrier in those transgenic mosquitoes, which was effective and protective against multiple strains and genotypes of DENV2 but not against other DENV serotypes. Unfortunately, Carb77 mosquitoes lost their anti-DENV2 resistance phenotype after 17 generations (G_{17}) in laboratory culture (Franz *et al.*, 2009). Apparently, expression of the antiviral effector gene was silenced in those transgenics that followed G_{17} whereas their eye markers were still highly expressed. This observation made us aware of so-called position effects, which can have long-term consequences regarding transgene stability and expression levels in *Ae. aegypti* (Henikoff, 1992; Elgin and Reuter, 2013). Apparently, certain TE integration loci are not ideal, due to the presence of interfering heterochromatic structures and/or promoters/enhancers of neighbouring genes, which could negatively affect effector gene expression. Choosing the identical transgene, which had been utilized in Carb77 mosquitoes, we then generated another series of transgenic *Ae. aegypti* (Franz *et al.*, 2014). As a result, we identified a transgenic line, Carb109, which strongly expressed the anti-DENV2 effector in midgut tissue, resulting in the same antiviral resistance phenotype as described for line Carb77. Importantly, after more than 10 years in laboratory culture (> 50 inbred generations), Carb109 females are still completely resistant to DENV2. Similar to Carb77, the genetic background of the Carb109 mosquitoes is the eye pigment-deficient Higgs White Eye (HWE) strain of *Ae. aegypti*, which is highly inbred and has been maintained in laboratory culture for over 20 years. We then introgressed the Carb109 transgene from the HWE genetic background into the genetic background of a genetically diverse laboratory strain (GDLS) consisting of ten *Ae. aegypti* populations collected in southern Mexico via six recurrent backcrosses followed by eye marker selection to identify transgenics. Similar to the original Carb109 mosquitoes, transgene-bearing GDLS were completely resistant to the virus when challenged with DENV2 via artificial bloodmeals. This demonstrated that the antiviral effector was similarly effective in different mosquito strains of *Ae. aegypti*.

In another germline transformation experiment, the DENV2 targeting IR effector was linked to the promoter of the *Ae. aegypti Vitellogenin 1* (*Vg1*) gene to trigger a DENV2-mediated RNAi response in the fat body of blood-fed females (A.W.E. Franz and K.E. Olson, unpublished data). In one of the generated transgenic lines, Vg40, the antiviral effector was strongly expressed at 10–24 h pbm as confirmed by northern blot analysis. However, during this narrow time window, orally acquired DENV2 has not yet disseminated from the female midgut. Thus, we provided females with an initial DENV2-containing bloodmeal to establish viral infection in the mosquito, followed by a non-infectious bloodmeal 5–6 days later to trigger the effector-mediated RNAi response in the fat body. At 24, 48 and 72 h post-second bloodmeal, mosquitoes were assayed for the presence of DENV2. Viral titres in whole-body Vg40 females reached similar levels as the non-transgenic HWE control. Furthermore, both Vg40 and control mosquitoes showed similar DENV2 titres in collected saliva samples. Thus, attempts to block DENV2 in secondary tissue surrounding the midgut, such as the fat body, did not block the progressive infection of the virus inside the mosquito.

The salivary glands represent the final tissue of the mosquito that needs to be infected by an arbovirus before it can be transmitted to a vertebrate host. Mathur *et al.* (2010) revealed that DENV2 replication can be blocked in the distal-lateral lobes of the female salivary glands utilizing the same IR construct, which had been used to block replication of the virus in the midgut. Transgenic mosquitoes were generated in which the IR effector was constitutively expressed from the bi-directional, salivary gland-specific *30K* promoter of *Ae. aegypti*. Five different lines were obtained, which showed salivary gland-specific expression of the IR effector and its processing into 21 bp siRNAs by the endogenous RNAi machinery. Oral challenge with DENV2 resulted in strong infection of mosquito carcasses (from which the salivary glands had been removed) at 15 days post-infection (dpi). However, salivary gland infection rates (27–40%) were significantly reduced in the transgenic mosquitoes in comparison with the non-transgenic control (62%). Importantly, two of the transgenic lines did not release any DENV2 in their saliva, while only 10–12% of the females belonging to the other three lines (compared with 60% of the non-transgenic control mosquitoes) had infectious virus in their saliva.

The long dsRNA effector strategy was also successfully applied against ZIKV when transgenic *Ae. aegypti* were generated in which the virus was silenced in the midgut epithelium. Earlier, the ZIKV genome had been analysed to identify multiple 400–500 nt long stretches of the viral RNA, which, when transiently expressed as dsRNA, would lead to maximal silencing of the homologous virus in midgut tissue (Magalhaes *et al.*, 2019). The region of the ZIKV RNA genome encoding parts of NS3/NS4a resulted in > 90% silencing efficiency of ZIKV in transient assays and was chosen for the generation of the transgenic mosquitoes (Williams *et al.*, 2020). The IR-molecule, about 450 bp in size, was linked to the *CPA* promoter as described before. A transgenic line, anti-ZIKV-NS3/4A IR, harbouring the construct strongly overexpressed the anti-ZIKV effector in the midgut epithelium of blood-fed females. This transgenic line was generated by site-specifically inserting the IR effector-harbouring transgene into a previously identified gene locus using CRISPR/Cas9 gene knock-in technology (see Ahmed and Wimmer, Chapter 5, this volume). In the context with another transgenic mosquito line, this gene locus, termed TIMP P4 (located in a non-coding region of chromosome 2q), was earlier identified to support robust transgene expression over subsequent generations (Dong *et al.*, 2017). We chose this locus to avoid the problem of TE-mediated, quasi-random transgene integration, which is prone to position effect variegation. Following oral challenge with ZIKV, complete inhibition of ZIKV replication was detected in 90% of the midguts obtained from the transgenic mosquitoes at 7 and 14 dpi, whereas over 50% of the midguts from the non-transgenic control exhibited high virus titres (Williams *et al.*, 2020). Furthermore, five in 30 transgenic females harboured ZIKV in their salivary glands, three of which also displayed saliva containing virus. By comparison, 59% of the controls had infected salivary glands and 33% of those released saliva containing virus.

Our results so far show that the long dsRNA effector strategy is highly efficient when targeting individual flaviviruses such as DENV2 or ZIKV. Early on, another point of interest was to see whether IR effector constructs could be designed to simultaneously target multiple viruses such as the four DENV serotypes. Therefore, a hybrid IR molecule was generated consisting of four cDNA fragments, about 300 bp long, that were fused together and originated from the genomes of DENV1, 2, 3 and 4. This IR effector molecule was placed under control for the *CPA* promoter and inserted into *mariner Mos1* TE. Transformation efficiencies with this transgene were very low in repeated experiments. Eventually, we obtained a transgenic *Ae. aegypti* line, which, unfortunately, did not show any level of resistance to any of the DENV serotypes (V. Barbosa-Solomieu and K.E. Olson, unpublished data). In another attempt, a transgene was assembled in which multiple IR sequences about 180 bp in length, each of them specific to different DENV serotypes, were linked together and

overexpressed from the *CPA* promoter. While antiviral resistance was initially observed in selected transgenic mosquito lines, the highly complex IR-effector structures likely were unstable when inserted into the *Ae. aegypti* genome using *mariner Mos1* as TE (V. Balaraman and A.W.E. Franz, unpublished data). Following a few generations of mosquito inbreeding, effector gene expression was silenced in those transgenic mosquitoes. Thus, the long dsRNA strategy to trigger the antiviral siRNA pathway looks highly efficient when targeting individual flaviviruses but so far has failed to target multiple viruses simultaneously.

22.3.3 Synthetic arbovirus-derived miRNA clusters that trigger antiviral RNAi in *Ae. aegypti*

Another synthetic antiviral effector type that has been explored in transgenic *Ae. aegypti* is synthetic miRNAs (Yen *et al*., 2018; Buchman *et al*., 2019). Yen and colleagues (2018) designed miRNAs to target the flavivirus DENV3 and the alphavirus CHIKV simultaneously in the same individual mosquito.

Both viruses are transmitted by *Ae. aegypti* and often co-circulate in tropical regions (Chahar *et al*., 2009; Rezza *et al*., 2014). A mosquito can acquire and transmit both viruses simultaneously (Furuya-Kanamori *et al*., 2016). Four regions from the DENV3 genome encoding the non-structural proteins NS2B, NS3 and NS5 (two targets) and six regions from the CHIKV genome encoding the non-structural proteins nsP1, nsP2, nsP3, nsP4 and the structural proteins E2 and E1 were selected as miRNA targets (Yen *et al*., 2018). Two promoters were chosen to control synthetic miRNA expression: the constitutive *PUb* promoter and the blood-meal-inducible *CPA* promoter (Edwards *et al*., 2000; Anderson *et al*., 2010). The class II *mariner Mos1* TE was used as the transgene insertion vector (Franz *et al*., 2006). Four different transgenic lines were established (Yen *et al*., 2018): PUb>4miR:DENV3, PUb>6miR:CHIKV, PUb>10miR and CPA>10miR

(the number in front of 'miR' indicates the number of clustered miRNA repeats). In the 10miR lines, the 4miR:DENV3 and 6miR:-CHIKV were fused into a single synthetic miRNA cluster. In all transgenic lines, synthetic miRNA expression was confirmed by quantitative PCR. In sugar-fed PUb>10miR females, antiviral miRNAs were detected in midgut and carcass tissues. At 24 h pbm, antiviral miRNA expression was increasingly detected in the midgut tissue of CPA>10miR females. Significantly fewer saliva samples obtained from transgenic mosquitoes overexpressing PUb>4miR:DENV3 or PUb>6miR:-CHIKV contained DENV3 (PUb>4miR:DENV3: 10%±4.5) or CHIKV (PUb>6miR:CHIKV: 8%±4.0) at 6 days post-infectious blood-meal in comparison with the non-transgenic control ('Orlando': 27%±6.4). Co-challenging of 24 PUb>10miR females and 24 CPA>10miR females with DENV3 and CHIKV (concentration of both viruses between 10^6 focus-forming units (ffu)/ml and 10^7 ffu/ml), resulted in 11% and 7%, respectively, positive saliva samples at 6 dpi, whereas 42% of the saliva samples of the non-transgenic control were positive for CHIKV and 25% of those were positive for DENV3. CHIKV and DENV3 antigens were also significantly less detectable (but not completely absent) in midguts and carcasses of the transgenic miRNA overexpressing mosquitoes. These results indicate that the mosquito's endogenous RNAi pathway recognized and processed the miRNA clusters causing resistance to DENV3 and CHIKV. Importantly, the work of Yen *et al*. (2018) represents an example in which two different arboviruses, CHIKV and DENV3, have been targeted and silenced by a single fusion effector molecule, although silencing of CHIKV was less efficient than that of DENV3.

In a following study, a polycistronic cluster of eight synthetic miRNA-like RNAs was designed to target the flavivirus, ZIKV, in transgenic mosquitoes (Buchman *et al*., 2019). The eight small RNAs targeted the three structural genes (C, prM-M and E) and three non-structural genes (NS1, NS2A and NS5) of the French Polynesia ZIKV strain H/PF/20)3. Synthetic small RNA expression was under control of the *CPA* promoter and

in this work the *piggyBac* TE was used as transgene insertion vector. One transgenic line (TZIKV-C) out of four that were generated was selected for further experiments. Using Illumina deep-sequencing, expression of non-guide and mature small RNA guide strands derived from five of the eight ZIKV-targeting synthetic small RNAs (small RNA # 1, 2, 4, 6 and 8) was detected in blood-fed females with transcript numbers ranging from 2 to 91 (mean: 25.7) per million values for mature small RNA guide strands. This indicated that the synthetic small RNAs were efficiently expressed from the transgene and processed by the endogenous RNAi machinery.

At 4 days post-oral challenge with ZIKV (strain FSS13025, Cambodia), administered by artificial bloodmeal, total RNA was extracted from the midguts of the transgenic (and non-transgenic control) females and assessed by quantitative RT-PCR for the presence of ZIKV genome copy equivalents. No ZIKV RNA was detected in midguts, carcasses and saliva (assayed by median tissue culture infectious dose [$TCID_{50}$]) of homozygous TZIKV-C mosquitoes at 4 dpi (*n*=32) and 14 dpi (*n*=46). However, ZIKV RNA was detectable in 88% (28/32) of the midguts analysed from heterozygous TZIKV-C females at 4 dpi and in 74% (29/39) of the midguts and carcasses analysed at 14 dpi. The virus was also detected in 74% of the saliva samples collected from the heterozygous transgenics at 14 dpi. Regardless, in comparison with the non-transgenic control, viral RNA copy equivalents were reduced by 2–3 $\log_{10}$ in tissues collected from the heterozygous TZIKV-C females. Thus, while homozygous transgenic mosquitoes were completely refractory to ZIKV infection, the authors claimed that the low-level intensity of infection in the heterozygous females would be insufficient to productively transmit the virus to a new vertebrate host, thereby effectively interrupting the viral disease cycle. This was confirmed when allowing transgenic mosquitoes, which had been challenged with ZIKV, to feed on immune-compromised (Stat1$^{-/-}$) mice (6–10 mosquitoes per mouse). While all mice that were exposed to control mosquitoes exhibiting high virus titres eventually succumbed to ZIKV infection, there was no mortality observed among those mice that were exposed to the transgenic mosquitoes that had been challenged with virus.

22.3.4 Antiviral effectors based on ribozymes to degrade arboviral RNA genomes

Design and function of hammerhead ribozymes

Ribozymes are self-catalytic small RNA molecules that cleave their RNA targets at specific recognition sites. A specific group of these, the hammerhead ribozymes (hRz), were initially identified among plant viroids and plant virus satellite RNAs (Kiefer *et al.*, 1982; Buzayan *et al.*, 1986; Hutchins *et al.*, 1986; Prody *et al.*, 1986). Previously, hRz have been engineered to act as synthetic antiviral effector molecules by inhibiting the replication of human pathogenic viruses, including human immunodeficiency virus (HIV) (Sun *et al.*, 1995; Jackson *et al.*, 1998), hepatitis B virus (HBV) (von Weizsacker *et al.*, 1992; Weinberg *et al.*, 2000) and hepatitis C virus (HCV) (Lieber *et al.*, 1996). hRz are capable of identifying and cleaving RNA targets as small as 15 nt (typically 18–19 nt) in length, allowing highly conserved sequences to be targeted. The antiviral effect of hRz is mediated through the catalytic activity of the molecule itself in the presence of magnesium ions and does not require additional host factors. Importantly, the multiple turnover kinetics of hRz allow a single hRz molecule to cleave multiple viral RNAs in the cell cytoplasm, which is an ideal situation to tackle viral RNAs at their onset of replication. Most natural hRz consist of a central conserved core RNA sequence flanked by three double-stranded regions with relaxed sequence requirements (helices I, II and III), two of which are capped by short loops. In an engineered (*trans*-cleaving) hRz, target RNA cleavage occurs following the pairing of the 5′ helix I and 3′ helix III arms of the hRz with a complementary (3′ to 5′ orientation) sequence motif of the target RNA (Fig. 22.2). The catalytic core of the hRz, represented by helix II, then cleaves the target RNA at the 3′

end of a 5′-NUH-3′ triplet motif (N = A, C, G, or T; H = A, C, or U) within the target sequence (Blount and Uhlenbeck, 2002; Nawtaisong *et al*., 2009). The overall catalytic potential of a hRz is dependent on the concentration of stable hRz molecules in a cell, an effective co-localization of hRz and target RNA, and the accessibility of the target RNA's cleavage site (which should not be obscured due to secondary structures). Linking an hRz to a powerful Pol III promoter such as tRNA[val] helped to ensure the overexpression of a high concentration of hRz molecules and their cytoplasmatic translocation.

Fourteen different hRz were designed to cleave various sequence motifs of the DENV2 (strain New Guinea C) RNA in infected
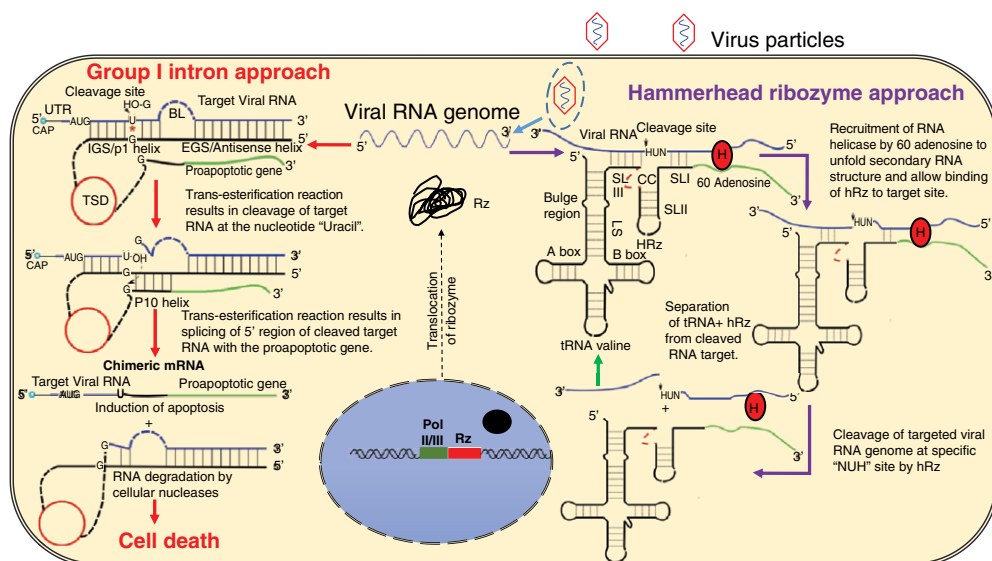
Fig. 22.2. Schematic representation of Group I Intron (GrpI) and hammerhead ribozyme (hRz) mediated inhibition of virus replication in mammalian and mosquito cells. GrpI are ribozymes consisting of an external (EGS) and an internal guide sequence (IGS), a *trans*-splicing domain and a 3′ exon (which can be derived from a pro-apoptotic gene). GrpI are expressed from Pol II promoters such as *Drosophila actin 5c*. In the cytoplasm, GrpI catalyse RNA cleavage in two *trans*-esterification steps. In the first step (TES-1), guanosine-mediated *trans*-esterification reaction results in cleavage of the viral target RNA downstream of the uracil position. In the second step (TES-2), *trans*-esterification is initiated by the free hydroxyl group of uracil attacking the phosphate group of guanosine in the upstream region of the P10 helix, which is linked to the 3′ exon. This reaction leads to ligation of the proximal region of the cleaved target RNA to the 3′ exon encoding a pro-apoptotic gene such as ΔN bax.

hRz are expressed from Pol III promoters. In the cytoplasm, hRz-mediated viral RNA cleavage requires two steps: the first step involves binding of stem loops I and III of hRz to the target viral RNA; the second step involves cleavage downstream of the NUH triplet (N = any nucleotide, U = Uracil, H = any nucleotide except guanine). After cleavage of the target RNA, hRz dislodges and then moves to the next target RNA, thereby following multiple turnovers.

Abbreviations: UTR, untranslated region; EGS, external guide sequence; IGS, internal guide sequence; BL, bulge loop; TSD, *trans*-splicing domain; TES, transesterification reaction; Rz, ribozyme (hammerhead ribozyme or Group I intron); Pol II/III, polymerase II or III promoter; CAP, 5′methyl guanosine; SL I, II, III, stem loop I, II III; CC, catalytic core; LS, linker sequence; hRz, hammerhead ribozyme; H, RNA helicase.

[Fig. 22.2 was published in *Current Opinion in Insect Science* 8: Alexander W.E. Franz, Velmurugan Balaraman, Malcolm J. Fraser Jr, Disruption of dengue virus transmission by mosquitoes, pp. 88–96, copyright Elsevier 2015 (with permission).]

Ae. albopictus C6/36 cells (Nawtaisong *et al*., 2009). Each of the engineered hRz was linked to the tRNA^{Val} promoter and contained a 3′ terminal poly-A$_{60}$ tail to recruit the unwinding activity of an endogenous RNA helicase (Craig *et al*., 1998). A lentivirus-based expression system was used to transduce the mosquito cells with the various hRz, whose expression was confirmed by RT-PCR. Four of the 14 different hRz were found to significantly suppress DENV2 replication in the infected cells as viral titres were reduced by up to two orders of magnitude (from 1.9×10^6 TCID$_{50}$ titre in the non-transduced cells down to 2.5×10^4 TCID$_{50}$ titre in ribozyme-expressing cell line hRz #11), which was confirmed at the viral RNA level by quantitative RT-PCR.

In another study, transgenic *Ae. aegypti* were generated that would constitutively overexpress hRz engineered to cleave the RNA genome of the CHIKV vaccine strain 181/25 (Mishra *et al*., 2016). Two different hRz were generated, one of which (hRz #9) targeted the viral subgenomic RNA promoter, while the other one (hRz #14) was targeting the E1 protein-encoding region of the viral RNA. Each hRz was placed under control of the tRNA^{val} Pol III promoter and a poly-A$_{60}$ tail was added to the 3′ end of the expression construct. The resulting cDNA constructs (each about 200 bp in size) were inserted into the *piggyBac* TE plasmid (Li *et al*., 2005). Each modified TE plasmid DNA was then co-injected with the helper plasmid *phspBAC* into 660–731 embryos (Mishra *et al*., 2016). In total, nine transgenic *Ae. aegypti* lines (seven hRz #9, two hRz #14) were obtained. Following ingestion of a CHIKV containing artificial bloodmeal, the virus was completely suppressed in seven of those nine different transgenic lines (zero CHIKV titre in 20–42 analysed females per line) at 7 dpi when mosquitoes were maintained as heterozygotes. This was confirmed by quantitative RT-PCR, *in situ* immuno-fluorescence assays (IFA), comparative TCID$_{50}$-IFA analysis, and analysis of CHIKV titres in individual mosquitoes and mosquito saliva. The study represents the first example in which an alphavirus (CHIKV) has been efficiently targeted and suppressed in mosquitoes by transgene-mediated overexpression of a specifically designed synthetic effector.

Design and function of group I introns

Trans-splicing group I introns (GrpI) are highly effective ribozymes for post-transcriptional RNA modification requiring a minimal target sequence of only 9 nt (Carter *et al*., 2010). The requirement of such a short target recognition sequence allows GrpI to target multiple related RNA viruses simultaneously as long as they have that particular short sequence motif in common. These introns cleave either single-stranded or perfectly paired dsRNAs at defined uracil positions and covalently join a 3′ exon tag to the end of the cleavage product. The *trans*-splicing reaction of the GrpI is derived from the natural *cis*-splicing reaction occurring along one continuous RNA molecule to join several exons. In case of *trans*-splicing, the intron and 3′ exon are located on the same RNA molecule whereas the 5′ exon may be located on a different molecule. The splicing reaction occurs in two different successive transesterification steps (Cech, 1991) (Fig. 22.2). The GrpI requires an accessible uracil nucleotide downstream of which the target sequence is cleaved. In a *trans*-splicing reaction, two separate segments of the intron are utilized: the internal guide sequence (IGS) as a part of the P1 helix and the external guide sequence (EGS), each of which is complementary to the target RNA sequence. The IGS is limited in size to a 9 bp sequence near the reactive uracil while the EGS can be of any length and forms a transient helix with the target RNA sequence downstream of the reactive uracil (Köhler *et al.,* 1999).

Several anti-DENV1-4 Group I *trans*-splicing introns (αDENV-GrpI) were designed to target via *trans*-splicing two different uracil bases (U143 and U132) in the highly conserved 5′-3′ cyclization sequence (CS) motif found in all four DENV serotypes and required for viral RNA replication (Hahn *et al*., 1987; Alvarez *et al*., 2005; Carter *et al*., 2010). This region is positioned within the capsid coding sequence at nucleotide positions C131-G151 and contains several uracil targets for the *trans*-splicing

reaction. Each αDENV-GrpI was constructed to include a 3′ firefly luciferase (FL) open reading frame (ORF) that permitted a quantitative assessment of splicing activity (Carter *et al*., 2010). Co-transfection assays for FL activity were performed in *Drosophila* S2 or *Ae. aegypti* Aag2 cells. An αDENV-GrpI 9v1 containing a 9 bp P1 helix and a 9 nt antisense EGS was designed to effectively *trans*-splice all known DENV1-4 sequences. Following its transfection into *Ae. aegypti* Aag2 cells or its constitutive expression in transformed *Ae. albopictus* C6/36 cells, GrpI 9v1 efficiently cleaved viral RNA at position U143 and *trans*-spliced the genomic RNA of DENV2. αDENV-GrpI versions 96v1, 96v3 and 96v4 contained an extended 96 nt antisense EGS which was designed to target DENV2 (strain New Guinea C). The three versions differed in their P10 helix architecture: 96v1 had a 6 bp P10 helix with no wobble base, and a standard P1 helix including the required wobble base; 96v3 differed from 96v1 in the deletion of 3 bp between the P10 helix and the catalytic core, while 96v4 incorporated a wobble base pairing downstream of the 3′ exon splice-site. Based on luciferase assays, two of these engineered GrpI, designated 9v1 and 96v4, yielded the highest quantities of *trans*-splice products compared with the other αDENV-GrpI variants. Furthermore, as shown by $TCID_{50}$-IFA analyses, αDENV-GrpI 9v1 and 96v4 activities suppressed DENV2 titres in Aag2 cells by 2 log_{10} and 3 log_{10}, respectively, while αDENV-GrpI 9v1, designed to target all four DENV serotypes, also suppressed the replication of DENV1, 3 and 4 serotypes. These results validated αDENV-GrpI introns as potent antiviral effector constructs that are able to suppress the infection of mosquito cells and tissues with any of the four DENV serotypes.

In a further study, the uracil 143 targeting αDENV-GrpI 9v1 was modified to catalyse *trans*-splicing of the 5′ CS region of DENV1-4 genomic RNAs to a ΔN Bax 3′ exon, which would induce apoptotic cell death upon infection (Carter *et al*., 2014). The insertion of a UAA stop codon in the *trans*-splicing domain of the intron prevented premature expression of the ΔN Bax 3′ exon that would otherwise induce apoptosis in non-infected cells. Sequences from the ΔN Bax 3′ exon displaced the distal portion of the P1 helix of the GrpI to form the P10 helix, which then allowed the second transesterification step to proceed, resulting in the ligation of the targeted DENV sequence and ΔN Bax. Following DENV1-4 infection, αDENV-U143-ΔN Bax targeted and cleaved DENV genomes at position uracil 143, thereby forming a chimeric mRNA consisting of the 5′ cap, 5′ UTR, 143 nt of the DENV capsid (DCA) coding sequence, and the 3′ ΔN Bax exon. The expressed DCA-ΔN Bax fusion protein induced apoptosis to block any productive virus infection as confirmed by Annexin V staining, caspase 3 assays and DNA ladder observations. Quantification of DENV1-4 infection in C6/36 cells at 4 dpi via $TCID_{50}$-IFA assays revealed that the co-expression of the ΔN Bax 3′ exon further enhanced the suppression of DENV1-4 (up to 5 log_{10}) in the infected cells by 2.5 log_{10}. This death-upon-infection strategy would also prevent the development of viral escape mutants that otherwise could perhaps avoid GrpI-mediated cleavage of their viral genomes.

Another development was the combination of DENV1-4 and CHIKV-specific internal and external guide sequences along with a corresponding interacting P10 sequence in a single *trans*-splicing GrpI (Carter *et al*., 2015). EGS, IGS and P10 helix-forming sequences derived from the highly conserved nsP1 encoding region of the CHIKV (strain: 181/25) RNA were inserted into the anti-DENV 9v1 construct (Carter *et al.,* 2010, 2014), allowing the targeting of genomic CHIKV RNA as well as DENV1-4 genomic RNAs by a single antiviral GrpI, which was coupled to the apoptosis-inducing ΔN Bax 3′ exon. Clonal C6/36 cell lines expressing CHIKV/DENVv1-ΔN Bax GrpI initiated a 10× greater level of apoptosis than negative control cells based on effector caspase activity. Furthermore, following infection with either CHIKV or DENV1-4, all six clonal C6/36 cell lines stably expressing CHIKV/DENVv1-ΔN Bax GrpI completely suppressed replication of either virus, resulting in no detectable virus titres as measured by $TCID_{50}$-IFA.

22.4 Single-chain Variable Fragments as Antiviral Effectors that Block Arboviral Proteins in *Ae. aegypti*

Single-chain variable fragments (scFv) that are transgenically overexpressed in a specific tissue of a mosquito represent a different antiviral effector type, which does not target and degrade the viral RNA genome of an arbovirus but instead blocks the viral infection cycle inside the cell at the protein level. This approach has been chosen to generate transgenic resistance against all four DENV serotypes in *Ae. aegypti* (Buchman *et al.*, 2020). This is a significant accomplishment representing the first example of an effector strategy that successfully generated simultaneous resistance to all four DENV serotypes in mosquitoes. The scFv was derived from a human monoclonal antibody, 1C19, which broadly neutralizes DENV1-4 (Smith *et al.*, 2013). DNA sequences for the 1C19 variable heavy and light chains were obtained from hybridoma cells expressing the human monoclonal antibody. The DNA sequence encoding a 15 amino acid glycine-serine repeat [G(4)S]3 linker was used to link the antibodies' VH and VL genes together (Yusakul *et al.*, 2016). The complete scFv cDNA was then cloned downstream of the *CPA* promoter sequence and inserted into the *piggyBac* TE used as transgene insertion vector (Buchman *et al.*, 2020). Three of the generated transgenic mosquito lines, TADV-A, TADV-B and TADV-C, were selected for further characterization. Increased 1C19 scFv expression was observed in midguts of blood-fed transgenic females at 24 h pbm. Western blot analysis of midgut protein extracts that were obtained from these females confirmed the binding of 1C19 scFv to DENV1-4 protein. Following oral challenge with DENV1-4 using artificial bloodmeals, homozygous TADV-A females were completely refractory to any of the DENV serotypes. As assayed by quantitative RT-PCR using DENV serotype-specific primers binding to NS5, there was no infection detectable among any midgut samples (*n*=28–35 for each virus serotype) obtained at 4 dpi from

homozygous females, which had been orally challenged with either DENV serotype. Similarly at 14 dpi, there was no DENV1-4 replication detectable in midguts or carcasses and none of the saliva samples (*n*=28–30 for each virus serotype) were positive for virus as tested by TCID$_{50}$. However, in heterozygous TADV-A mosquitoes, orally acquired DENV2 was detected in 85% (35/41) of the females tested although these mosquitoes exhibited significantly lower (about 3 log$_{10}$) viral RNA copy equivalents than the wild-type control. Similarly, 83% (25/30) of heterozygous TADV-A mosquitoes showed detectable amounts of DENV2 in their saliva, with TCID$_{50}$ titres being significantly reduced in comparison with the non-transgenic control. Viral detection data looked similar for the other three DENV serotypes in heterozygous TADV-A females. Thus, in homozygous but not in heterozygous scFv-expressing females, the scFv effector completely shut down the infection of midgut tissue with DENV1-4. Consequently, these mosquitoes were unable to transmit any of the DENV serotypes.

22.5 Conclusions and Outlook

Great progress has been made in the past 5 years regarding the design and concepts of synthetic antiviral effector molecules to block the transmission of arboviruses in transgenic *Ae. aegypti*. Effector molecules that target and cleave the viral RNA of infecting viruses include long dsRNAs and synthetic miRNAs, both of which rely on recognition and processing by the endogenous RNAi machinery of the mosquito. Long dsRNA effectors are relatively easy to design and assemble. Based on the length of the dsRNA molecule these effectors produce, they are relatively insensitive to viral mutations affecting single nucleotides in their target regions. However, based on the outcomes of repeated efforts, it can be concluded that the long dsRNA strategy might not be suitable for multiplexing, i.e., targeting multiple arboviruses simultaneously. Synthetic miRNAs form a highly effective alternative to long

dsRNAs. Although more complicated to design, polycistronic clusters of synthetic miRNAs can be assembled to target different regions of the viral genome of a particular virus or the RNA genomes of multiple viruses simultaneously. Synthetic miRNAs were successfully designed to generate transgenic resistance to ZIKV and DENV3 as well as to the alphavirus, CHIKV. Another effector strategy aiming at the degradation of viral RNAs is hRz and GrpI, both of which are complicated to design. hRz and GrpI, both recognizing and cleaving their target RNAs independently of any cellular antiviral pathway, look highly promising as antiviral effectors when designed to target DENV, ZIKV, or CHIKV. These effector types have been predominantly validated in cell culture and now need to be more thoroughly tested in mosquitoes. scFv represents an antiviral effector type that does not attack the viral RNA genome but instead blocks the assembled virion (or its assembly) inside the infected cell. This effector type, when transgenically overexpressed, is the first example in which all four DENV serotypes have been completely shut down in mosquitoes. Thus, scFvs seem to have a high potential as antiviral effectors, as long as strong monoclonal antibodies targeting the virus of interest are available, which are needed to serve as a 'genetic blueprint' for the scFv design.

Synthetic antiviral effectors have been overexpressed in three key tissues of the mosquito that are naturally infected with arboviruses: the midgut, the fat body and the salivary glands. It appears that overexpression in the midgut, the initial tissue that gets infected with an orally acquired arbovirus, is the most reliable and efficient approach to block transmission of an arbovirus by the mosquito.

A critically important factor affecting the performance of all effector types discussed here is the transgene integration site. With the exception of the transgenic mosquitoes targeting ZIKV generated by Williams *et al*. (2020), all antiviral effectors have been quasi-randomly inserted into the mosquito genome with the help of non-autonomous TEs such as *mariner Mos1* or *piggyBac*. Thus, several different lines arising from the same germline transformation experiment need to be established and comparatively tested for effector gene performance/antiviral resistance to identify the line with the optimal performance. Independent of eye marker expression levels, tissue-specific effector gene expression levels can vary substantially due to position effects. As previously observed, effector gene expression can cease after several generations of inbreeding of a transgenic line under laboratory conditions. Another potential problem leading to inheritable instabilities is that both *mariner Mos1* and *piggyBac* may promote multiple independent transgene integration events in different loci. Thus, a critical asset when generating transgenic mosquitoes to overexpress antiviral effectors is the knowledge of robust transgene insertion loci allowing strong effector gene performance. Using CRISPR/Cas9 as a site-specific transgene insertion tool, such loci can now be specifically targeted as shown by Williams *et al*. (2020), representing a further improvement of transgenic mosquito technology.

References

Adams, B. and Kapan, D.D. (2009) Man bites mosquito: understanding the contribution of human movement to vector-borne disease dynamics. *PLoS ONE* 4(8), e6763.

Alvarez, D.E., Lodeiro, M.F., Luduena, S.J., Pietrasanta, L.I. and Gamarnik, A.V. (2005) Long-range RNA–RNA interactions circularize the dengue virus genome. *Journal of Virology* 79(11), 6631–6643.

Anderson, M.A., Gross, T.L., Myles, K.M. and Adelman, Z.N. (2010) Validation of novel promoter sequences derived from two endogenous ubiquitin genes in transgenic *Aedes aegypti*. *Insect Molecular Biology* 19(4), 441–449.

Angleró-Rodríguez, Y.I., MacLeod, H.J., Kang, S., Carlson, J.S., Jupatanakul, N. and Dimopoulos, G. (2017) *Aedes aegypti* molecular responses to Zika virus: modulation of infection by the Toll and Jak/Stat immune pathways and virus host factors. *Frontiers in Microbiology* 8, 2050.

Barrows, N.J., Campos, R.K., Liao, K.C., Prasanth, K.R., Soto-Acosta, R. *et al.* (2018) Biochemistry and molecular biology of flaviviruses. *Chemical Reviews* 118, 4448–4482.

Beerntsen, B.T., Champagne, D.E., Coleman, J.L., Campos, Y.A. and James, A.A. (1999) Characterization of the Sialokinin I gene encoding the salivary vasodilator of the yellow fever mosquito, *Aedes aegypti. Insect Molecular Biology* 8(4), 459–467.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W. *et al.* (2013) The global distribution and burden of dengue. *Nature* 496, 504–507.

Black, W.C. 4th, Alphey, L. and James, A.A. (2011) Why RIDL is not SIT. *Trends in Parasitology* 27(8), 362–370.

Blitvich, B. (2008) Transmission dynamics and changing epidemiology of West Nile virus. *Animal Health Research Reviews* 9, 71–86.

Blount, K.F. and Uhlenbeck, O.C. (2002) The hammerhead ribozyme. *Biochemical Society Transactions* 30, 1119–1122.

Bowman, N.M., Akialis, K., Cave, G., Barrera, R., Apperson, C.S. and Meshnick, S.R. (2018) Pyrethroid insecticides maintain repellent effect on knock-down resistant populations of *Aedes aegypti* mosqui-toes. *PLoS ONE* 13(5), e0196410.

Buchman, A., Gamez, S., Li, M., Antoshechkin, I., Li, H.H. *et al.* (2019) Engineered resistance to Zika virus in transgenic *Aedes aegypti* expressing a polycistronic cluster of synthetic small RNAs. *Proceedings of the National Academy of Sciences USA* 116(9), 3656–3661.

Buchman, A., Gamez, S., Li, M., Antoshechkin, I., Li, H.H. *et al.* (2020) Broad dengue neutralization in mosquitoes expressing an engineered antibody. *PLoS Pathogens* 16(1), e1008103.

Buzayan, J.M., Gerlach, W.L. and Bruening, G. (1986) Satellite tobacco ringspot virus RNA: a subset of the RNA sequence is sufficient for autolytic processing. *Proceedings of the National Academy of Sciences USA* 83, 8859–8862.

Campbell, C.L., Black, W.C. 4th, Hess, A.M. and Foy, B.D. (2008) Comparative genomics of small RNA regulatory pathway components in vector mosquitoes. *BMC Genomics* 9, 425.

Carrera, J.P., Díaz, Y., Denis, B., Barahona de Mosca, I., Rodriguez, D. *et al.* (2017) Unusual pattern of chikungunya virus epidemic in the Americas, the Panamanian experience. *PLoS Neglected Tropical Diseases* 11, 2.

Carter, J.R., Keith, J.H., Barde, P.V., Fraser, T.S. and Fraser, M.J. Jr (2010) Targeting of highly conserved Dengue virus sequences with anti-Dengue virus trans-splicing group I introns. *BMC Molecular Biology* 11, 84.

Carter, J.R., Keith, J.H., Fraser, T.S., Dawson, J.L., Kucharski, C.A. *et al.* (2014) Effective suppression of dengue virus using a novel group-I intron that induces apoptotic cell death upon infection through conditional expression of the Bax C-terminal domain. *Virology Journal* 11, 111.

Carter, J.R., Taylor, S., Fraser, T.S., Kucharski, C.A., Dawson J.L. and Fraser, M.J. (2015) Suppression of the arboviruses dengue and Chikungunya using a dual-acting group-I intron coupled with conditional expression of the Bax C-terminal domain. *PLoS ONE* 10, e0139899.

Carvalho, F.D. and Moreira, L.A. (2017) Why is *Aedes aegypti* Linnaeus so successful as a species? *Neotropical Entomology* 46(3), 243–255.

Cech, T.R. (1991) RNA editing: world's smallest introns? *Cell* 64(4), 667–669.

Chahar, H.S., Bharaj, P., Dar, L., Guleria, R., Kabra, S.K. and Broor, S. (2009) Co-infections with chikungunya virus and dengue virus in Delhi, India. *Emerging Infectious Diseases* 15(7), 1077–1080.

Chang, C., Ortiz, K., Ansari, A. and Gershwin, M.E. (2016) The Zika outbreak of the 21st century. *Journal of Autoimmunology* 68, 1–13.

Cirimotich, C.M., Scott, J.C., Phillips, A.T., Geiss, B.J. and Olson, K.E. (2009) Suppression of RNA interference increases alphavirus replication and virus-associated mortality in *Aedes aegypti* mosquitoes. *BMC Microbiology* 9, 49.

Clem, R.J. (2016) Arboviruses and apoptosis: the role of cell death in determining vector competence. *Journal of General Virology* 97(5), 1033–1036.

Craig, A.W., Haghighat, A., Yu, A.T. and Sonenberg, N. (1998) Interaction of polyadenylate-binding protein with the eIF4G homologue PAIP enhances translation. *Nature* 392, 520–523.

Cui, Y., Grant, D.G., Lin, J., Yu, X. and Franz, A.W.E. (2019) Zika virus dissemination from the midgut of *Aedes aegypti* is facilitated by bloodmeal-mediated structural modification of the midgut basal lamina. *Viruses* 11(11), 1056.

Dong, S., Balaraman, V., Kantor, A.M., Lin, J., Grant, D.G. *et al.* (2017) Chikungunya virus dissemination from the midgut of *Aedes aegypti* is associated with temporal basal lamina degradation during bloodmeal digestion. *PLoS Neglected Tropical Diseases* 11(9), e0005976.

Edwards, M.J., Moskalyk, L.A., Donelly-Doman, M., Vlaskova, M., Noriega, F.G. *et al.* (2000) Characterization of a carboxypeptidase A gene from the mosquito, *Aedes aegypti. Insect Molecular Biology* 9(1), 33–38.

Elgin, S.C. and Reuter, G. (2013) Position-effect variegation, heterochromatin formation, and gene silencing in *Drosophila*. *Cold Spring Harbor Perspectives in Biology* 5(8), a017780.

Franz, A.W., Sanchez-Vargas, I., Adelman, Z.N., Blair, C.D., Beat,y B.J. *et al.* (2006) Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. *Proceedings of the National Academy of Sciences USA* 103(11), 4198–4203.

Franz, A.W., Sanchez-Vargas, I., Piper, J., Smith, M.R., Khoo, C.C. *et al.* (2009) Stability and loss of a virus resistance phenotype over time in transgenic mosquitoes harbouring an antiviral effector gene. *Insect Molecular Biology* 18(5), 661–672.

Franz, A.W., Sanchez-Vargas, I., Raban, R.R., Black, W.C. 4th, James, A.A. and Olson, K.E. (2014) Fitness impact and stability of a transgene conferring resistance to dengue-2 virus following introgression into a genetically diverse *Aedes aegypti* strain. *PLoS Neglected Tropical Diseases* 8(5), e2833.

Franz, A.W., Kantor, A.M., Passarelli, A.L. and Clem, R.J. (2015) Tissue barriers to arbovirus infection in mosquitoes. *Viruses* 7(7), 3741–3767.

Furuya-Kanamori, L., Liang, S., Milinovich, G., Soares Magalhaes, R.J., Clements, A.C. *et al.* (2016) Co-distribution and co-infection of chikungunya and dengue viruses. *BMC Infectious Diseases* 3, 16.

Gould, E., Pettersson, J., Higgs, S., Charrel, R. and de Lambalerrie, X. (2017) Emerging arboviruses: why today? *Open Health* 4, 1–13.

Gubler, D.J. (2002) The global emergence/resurgence of arboviral diseases as public health problems. *Archives of Medical Research* 33(4), 330–342.

Guzmán, C., Calderón, A., Mattar, S., Tadeu-Figuereido, L., Salazar-Bravo, J. *et al.* (2020) Ecoepidemiology of alphaviruses and flaviviruses. *Emerging and Reemerging Viral Pathogens* 2020, 101–125.

Guzman, M.G., Halstead, S.B., Artsob, H., Buchy, P., Farrar, J. *et al.* (2010) Dengue: a continuing global threat. *Nature Reviews Microbiology* 10, S7–S18.

Hahn, C.S., Hahn, Y.S., Rice, C.M., Lee, E., Dalgarno, L. *et al.* (1987) Conserved elements in the 3' untranslated region of flavivirus RNAs and potential cyclization sequences. *Journal of Molecular Biology* 198(1), 33–41.

Hahn, M.B., Eisen, L., McAllister, J., Savage, H.M., Mutebi, J.P. and Eisen, R.J. (2017) Updated reported distribution of *Aedes* (*Stegomyia*) *aegypti* and *Aedes* (*Stegomyia*) *albopictus* (Diptera: Culicidae) in the United States, 1995–2016. *Journal of Medical Entomology* 54(5), 1420–1424.

Halstead, S.B. and Aguiar, M (2016) Dengue vaccines: are they safe for travelers? *Travel Medicine and Infectious Diseases* 14(4), 378–383.

Harrington, L.C., Scott, T.W., Lerdthusnee, K., Coleman, R.C., Costero, A. *et al.* (2005) Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. *American Journal of Tropical Medicine and Hygiene* 72(2), 209–220.

Hegde, N.R. and Gore, M.M. (2017) Japanese encephalitis vaccines: immunogenicity, protective efficacy, effectiveness, and impact on the burden of disease. *Human Vaccines & Immunotherapeutics* 13(6), 1–18.

Henikoff, S. (1992) Position effect and related phenomena. *Current Opinion in Genetics and Development* 2(6), 907–912.

Herrera-Bojórquez, J., Trujillo-Peña, E., Vadillo-Sánchez, J., Riestra-Morales, M., Che-Mendoza, A. *et al.* (2020) Efficacy of long-lasting insecticidal nets with declining physical and chemical integrity on *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology* 57(2), 503–510.

Hutchins, C.J., Rathjen, P.D., Forster, A.C. and Symons, R.H. (1986) Self-cleavage of plus and minus RNA transcripts of avocado sunblotch viroid. *Nucleic Acids Research* 14, 3627–3640.

Jackson, W.H. Jr, Moscoso, H., Nechtman, J.F., Galileo, D.S., Garver, F.A. and Lanclos, K.D. (1998) Inhibition of HIV-1 replication by an anti-tat hammerhead ribozyme. *Biochemical and Biophysical Research Communications* 245, 81–84.

James, A.A. (2005) Gene drive systems in mosquitoes: rules of the road. *Trends in Parasitology* 21(2), 64–67.

Jasinskiene, N., Coates, C.J., Benedict, M.Q., Cornel, A.J., Rafferty, C.S. *et al.* (1998) Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the *Hermes* element from the housefly. *Proceedings of the National Academy of Sciences USA* 95(7), 3743–3747.

Jose, J., Snyder, J.E., Richard, J. and Kuhn, R.J. (2009) A structural and functional perspective of alphavirus replication and assembly. *Future Microbiology* 4, 837–856.

Kantor, A.M., Grant, D.G., Balaraman, V., White, T.A. and Franz, A.W.E. (2018) Ultrastructural analysis of chikungunya virus dissemination from the midgut of the yellow fever mosquito, *Aedes aegypti. Viruses* 10(10), 571.

Kek, R., Hapuarachchi, H.C., Chung, C.Y., Humaidi, M.B., Razak, M.A.B.A. *et al.* (2014) Feeding host range of *Aedes albopictus* (Diptera: Culicidae) demonstrates its opportunistic host-seeking behavior in rural Singapore. *Journal of Medical Entomology* 51(4), 880–884.

Khoo, C.C., Piper, J., Sanchez-Vargas, I., Olson, K.E. and Franz, A.W. (2010) The RNA interference pathway affects midgut infection- and escape barriers for Sindbis virus in *Aedes aegypti*. *BMC Microbiology* 10, 130.

Khoo, C.C., Doty, J.B., Heersink, M.S., Olson, K.E. and Franz, A.W. (2013) Transgene-mediated suppression of the RNA interference pathway in *Aedes aegypti* interferes with gene silencing and enhances Sindbis virus and dengue virus type 2 replication. *Insect Molecular Biology* 22(1), 104–114.

Kiefer, M.C., Daubert, S.D., Schneider, I.R. and Bruening, G. (1982) Multimeric forms of satellite of tobacco ringspot virus RNA. *Virology* 121, 262–273.

Köhler, U., Ayre, B.G., Goodman, H.M. and Haseloff, J. (1999) Trans-splicing ribozymes for targeted gene delivery. *Journal of Molecular Biology* 285(5), 1935–1950.

Kokoza, V., Ahmed, A., Cho, W.L., Jasinskiene, N., James, A.A. and Raikhel, A. (2000) Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. *Proceedings of the National Academy of Sciences USA* 97(16), 9144–9149.

Lambrechts, L., Scott, T.W. and Gubler, D.J. (2010) Consequences of the expanding global distribution of *Aedes albopictus* for dengue virus transmission. *PLoS Neglected Tropical Diseases* 4(5), e646.

Li, X., Harrell, R.A., Handler, A.M., Beam, T., Hennessy, K. and Fraser, M.J. Jr (2005) *PiggyBac* internal sequences are necessary for efficient transformation of target genomes. *Insect Molecular Biology* 14, 17–30.

Lieber, A., He, C.Y., Polyak, S.J., Gretch, D.R., Barr, D. and Kay, M.A. (1996) Elimination of hepatitis C virus RNA in infected human hepatocytes by adenovirus-mediated expression of ribozymes. *Journal of Virology* 70, 8782–8791.

Liu-Helmersson, J., Brännström, Å., Sewe, M.O., Semenza, J.C. and Rocklöv, J. (2019) Estimating past, present, and future trends in the global distribution and abundance of the arbovirus vector *Aedes aegypti* under climate change scenarios. *Frontiers in Public Health* 7, 148.

Lobo, N., Li, X. and Fraser, M.J. Jr (1999) Transposition of the *piggyBac* element in embryos of *Drosophila melanogaster*, *Aedes aegypti* and *Trichoplusia ni*. *Molecular Genetics and Genomics* 261(4–5), 803–810.

Magalhaes, T., Bergren, N.A., Bennett, S.L., Borland, E.M., Hartman, D.A. *et al.* (2019) Induction of RNA interference to block Zika virus replication and transmission in the mosquito *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* 111, 103169.

Mathur, G., Sanchez-Vargas, I., Alvarez, D., Olson, K.E., Marinotti, O. and James, A.A. (2010) Transgene-mediated suppression of dengue viruses in the salivary glands of the yellow fever mosquito, *Aedes aegypti*. *Insect Molecular Biology* 19, 753–763.

Medhora, M.M., MacPeek, A.H. and Hartl, D.L. (1988) Excision of the *Drosophila* transposable element mariner: identification and characterization of the Mos factor. *EMBO Journal* 7(7),2185–2189.

Mishra, P., Furey, C., Balaraman, V. and Fraser, M.J. (2016) Antiviral hammerhead ribozymes are effective for developing transgenic suppression of chikungunya virus in *Aedes aegypti* mosquitoes. *Viruses* 8(6), 163.

Mordecai, E.A., Ryan, S.J., Caldwell, J.M., Shah, M.M. and LaBeaud, A.D. (2020) Climate change could shift disease burden from malaria to arboviruses in Africa. *Lancet Planet Health* 4(9), e416–e423.

Myles, K.M., Wiley, M.R., Morazzani, E.M. and Adelman, Z.N. (2008) Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. *Proceedings of the National Academy of Sciences USA* 105(50), 19938–19943.

Nash, D., Mostashari, F., Fine, A., Miller, J., O'Leary, D. *et al.* (2001) West Nile Outbreak Response Working Group. The outbreak of West Nile virus infection in the New York City area in 1999. *New England Journal of Medicine* 344(24), 1807–1814.

Nawtaisong, P., Keith, J., Fraser, T., Balaraman, V., Kolokoltsov, A. *et al.* (2009) Effective suppression of Dengue fever virus in mosquito cell cultures using retroviral transduction of hammerhead ribozymes targeting the viral genome. *Virology Journal* 6, 73.

O'Brochta, D.A. and Atkinson, P.W. (1996) Transposable elements and gene transformation in non-drosophilid insects. *Insect Biochemistry and Molecular Biology* 26, 739–753.

Olson, K.E, and Blair, C.D. (2015) Arbovirus–mosquito interactions: RNAi pathway. *Current Opinion in Virology* 15, 119–126.

O'Neal, S.T., Samuel, G.H., Adelman, Z.N. and Myles, K.M. (2014) Mosquito-borne viruses and suppressors of invertebrate antiviral RNA silencing. *Viruses* 6(11), 4314–4331.

Ooi, E.E., Goh, K.T. and Gubler, D.J. (2006) Dengue prevention and 35 years of vector control in Singapore. *Emerging Infectious Diseases* 12(6), 887–893.

Pastula, D.M., Smith, D.E., Beckham, J.D. and Tyler, K.L. (2016) Four emerging arboviral diseases in North America: Jamestown Canyon, Powassan, chikungunya, and Zika virus diseases. *Journal of Neurovirology* 22, 257–260.

Pereira Cabral, B., da Graça Derengowski Fonseca, M. and Mota, F.B. (2019) Long term prevention and vector control of arboviral diseases: what does the future hold? *International Journal of Infectious Diseases* 89, 169–174.

Pratt, A.J. and MacRae, I.J. (2009) The RNA-induced silencing complex: a versatile gene-silencing machine. *Journal of Biological Chemistry* 284(27), 17897–17901.

Prody, G.A., Bakos, J.T., Buzayan, J.M., Schneider, I.R. and Bruening, G. (1986) Autolytic processing of dimeric plant virus satellite RNA. *Science* 231, 1577–1580.

Rezza, G., El-Sawaf, G., Faggioni, G., Vescio, F., Al Ameri, R. *et al.* (2014) Co-circulation of dengue and chikungunya viruses, Al Hudaydah, Yemen, 2012. *Emerging Infectious Diseases* 20(8), 1351–1354.

Roth, A., Mercier, A., Lepers, C., Hoy, D., Duituturaga, S. *et al* (2014) Concurrent outbreaks of dengue, chikungunya and zika virus infections—an unprecedented epidemic wave of mosquito-borne viruses in the Pacific 2012–2014. *Eurosurveillance* 19, 20929.

Rückert, C, and Ebel, G.D. (2018) How do virus–mosquito interactions lead to viral emergence? *Trends in Parasitology* 34(4), 310–321.

Sanchez-Vargas, I., Travanty, E.A., Keene, K.M., Franz, A.W., Beaty, B.J. *et al.* (2004) RNA interference, arthropod-borne viruses, and mosquitoes. *Virus Research* 102(1), 65–74.

Sánchez-Vargas, I., Scott, J.C., Poole-Smith, B.K., Franz, A.W., Barbosa-Solomieu, V. *et al.* (2009) Dengue virus type 2 infections of *Aedes aegypti* are modulated by the mosquito's RNA interference pathway. *PLoS Pathogens* 5(2), e1000299.

Schrama, M., Hunting, E.R., Beechler, B.R., Guarido, M.M., Govender, G. *et al.* (2020) Human practices promote presence and abundance of disease-transmitting mosquito species. *Science Reports* 10(1).

Scott, T.W. and Takken, W. (2012) Feeding strategies of anthropophilic mosquitoes result in increased risk of pathogen transmission. *Trends in Parasitology* 28(3), 114–121.

Simmons, C.P., Farrar, J.J., Nguyen, v.V. and Wills, B. (2012) Dengue. *New England Journal of Medicine* 366(15), 1423–1432.

Smith, S.A., de Alwis, A.R., Kose, N., Harris, E., Ibarra, K.D. *et al.* (2013) The potent and broadly neutralizing human dengue virus-specific monoclonal antibody 1C19 reveals a unique cross-reactive epitope on the bc loop of domain II of the envelope protein. *mBio* 4(6), e00873-13.

Sukhralia, S., Verma, M., Gopirajan, S., Dhanaraj, P.S., Lal, R. *et al.* (2019) From dengue to Zika: the wide spread of mosquito-borne arboviruses. *European Journal of Clinical Microbiology and Infectious Diseases* 38, 3–14.

Sun, L.Q., Wang, L., Gerlach, W.L. and Symonds, G. (1995) Target sequence-specific inhibition of HIV-1 replication by ribozymes directed to tat RNA. *Nucleic Acids Research* 23, 2909–2913.

Theiler, M. and Smith, H.H. (1937) The use of the yellow fever virus modified by in vitro cultivation for human immunization. *Journal of Experimental Medicine* 65(6), 787–800.

von Weizsacker, F., Blum, H.E. and Wands, J.R. (1992) Cleavage of hepatitis B virus RNA by three ribozymes transcribed from a single DNA template. *Biochemical and Biophysical Research Communications* 189, 743–748.

Weaver, S.C. (2014) Arrival of chikungunya virus in the New World: prospects for spread and impact on public health. *PLoS Neglected Tropical Diseases* 8(6), e2921.

Weaver, S. and Barrett, A. (2004) Transmission cycles, host range, evolution and emergence of arboviral disease. *Nature Reviews Microbiology* 2, 89–801.

Weaver, S.C. and Reisen, W.K. (2010) Present and future arboviral threats. *Antiviral Research* 85(2), 328–345.

Weinberg, M., Passman, M., Kew, M. and Arbuthnot, P. (2000) Hammerhead ribozyme-mediated inhibition of hepatitis B virus × gene expression in cultured cells. *Journal of Hepatology* 33, 142–151.

Williams, A.E., Sanchez-Vargas, I., Reid, W.R., Lin, J., Franz, A.W.E. and Olson, K.E. (2020) The antiviral small-interfering RNA pathway induces Zika virus resistance in transgenic *Aedes aegypti*. *Viruses* 12(11), 1231.

Yen, P.S., James, A.A., Li, J.C., Chen, C.H. and Failloux, A.B. (2018) Synthetic miRNAs induce dual arboviral-resistance phenotypes in the vector mosquito *Aedes aegypti*. *Communications Biology* 1, 11.

Yusakul, G., Sakamoto, S., Pongkitwitoon, B., Tanaka, H. and Morimoto, S. (2016) Effect of linker length between variable domains of single chain variable fragment antibody against daidzin on its reactivity. *Bioscience, Biotechnology, and Biochemistry* 80(7), 1306–1312.

23   Self-Limiting Insects for Pest Management

Neil I. Morrison*

Oxitec Limited, 71 Innovation Drive, Oxford OX14 4RQ, United Kingdom

---

23.1   Re-engineering the Sterile Insect Technique

The sterile insect technique (SIT) (Knipling, 1955; Dyck *et al.*, 2005) has been used successfully against a range of agricultural pest insects (see Scott *et al.*, Chapter 17, this volume). The method depends on the release of sterile insects (Box 23.1), which will mate with their wild counterparts, thereby reducing the reproductive potential of the wild population. If over time enough wild insects mate with sterile rather than fertile partners, the target population will decline. Sterile insect methods have several key features that may make them desirable in combination with, or as alternatives to, other pest control methods. The released sterile insects impact pest populations through mating and will mate only with sexually compatible partners; sterile insect methods are therefore strikingly species-specific with minimal off-target effects. Furthermore, the sterile insects will actively and specifically seek out conspecific mates; this allows low-density and cryptic populations to be effectively targeted.

Elimination (reduction to zero) of target pest populations is possible by this method, but may not be feasible or even desirable in every case. Examples of successful SIT programmes include the elimination of the New World screwworm *Cochliomyia hominivorax* from North and Central America (Klassen and Curtis, 2005) and from Libya (Lindquist *et al.*, 1992; Vargas *et al.*, 1994), and several against various tephritid fruit flies, especially the Mediterranean fruit fly *Ceratitis capitata* (Klassen and Curtis, 2005). Non-dipteran targets have included the pink bollworm *Pectinophora gossypiella* (Vanderplank, 1944; Grefenstette *et al.*, 2009), codling moth *Cydia pomonella* (Bloem *et al.*, 2005) and painted apple moth *Teia anartoides* (Suckling *et al.*, 2007) (see Scott *et al.*, Chapter 17, this volume).

Despite these many successes and its attractive features as a clean, demonstrably sustainable, species-specific control method usable on large and small scales, SIT remains restricted to a small number of pests and programmes. While this is partly related to aspects of the method – species-specificity, while environmentally desirable, is problematic when multiple pests attack a crop or vector a pathogen – it is also partly due to limitations that can potentially be overcome or mitigated through genetics. This article discusses some genetics-based improvements

---

*Email: Neil.Morrison@oxitec.com

Box 23.1. 'Sterility' (adapted from Alphey *et al.*, 2010)

Use of the term 'sterility' sometimes causes confusion. Despite the name 'sterile insect technique', the insects used in SIT are not strictly sterile, in the sense of agametic sterility. Rather, they produce gametes and are capable of mating, but some or all of the progeny of mating between the sterile insects and wild insects are inviable. At least for sterile males, production of gametes is likely to be important for their use in population control, due to post-copulatory effects such as sperm competition. If an individual female mates both a fertile and a sterile male, both types of sperm will typically compete to fertilize eggs – males that do not produce sperm will likely lose in such a competition. For species where the females mate only once this may be less of a problem, though in such circumstances increased remating might provide a simple form of behavioural resistance to SIT.

Several sterilizing methods are available. Here we use the terms 'sterile', 'sterility' and the like, for all of these methods, and the term 'SIT' to encompass the use of any or all of them. These include the following.

- Radiation, which is used in all current agricultural programmes, generates random dominant lethal mutations in the affected gametes.
- *Wolbachia*-induced cytoplasmic incompatibility, in which sperm from *Wolbachia*-infected males fail to function correctly after fertilizing eggs from uninfected females ('incompatible insect technique', IIT).
- Recombinant DNA methods, for example the use of engineered repressible dominant lethal mutations, that lead to some or all of the progeny of any cross involving an engineered 'sterile' parent being non-viable unless provided with a suitable antidote (repressor) to the lethal genetic system. In one embodiment of this system, the lethal effect is female-specific, so that only female progeny die.

Other methods have also been used historically, including chemosterilants (Breeland *et al.*, 1974) or incompatible matings, through the use of either sibling species (Vanderplank, 1944; Davidson *et al.*, 1970) or else the use of artificially induced chromosome rearrangements (Whitten and Foster, 1975; Foster *et al.*, 1988).

---

that are available with current technology, and how they are currently being implemented. These and related improvements – collectively termed 'self-limiting insects' - are designed to retain those key strengths of sterile insect methods, but make them much more practical and cost-effective against a wider range of pest species and in a wider range of contexts than is currently the case, and may thereby dramatically improve our ability to protect people, livestock and crops from important pests. Self-limiting insects have now been deployed in the field in multiple countries, delivering highly effective suppression of target pest populations. This approach is now on the brink of delivering large-scale impact against pests important to public health and agriculture.

23.2   Sterile Insects and Genetic Control

Genetic control methods use genetic elements, vertically transmitted via mating, to achieve their effects. This may be contrasted with other control methods such as the use of toxic chemicals, mating-disrupting pheromones, microbial pathogens, or the release of predators or parasitoids. These genetic elements may be stably present in the insect's genome, or present extra-chromosomally, for example episomally, or in plastids or other vertically transmitted elements such as *Wolbachia*. They may alternatively be transiently present. For example, in classical SIT, dominant-lethal mutations are induced by radiation; these are not present in the mass-reared strain but induced just prior to release, and are expected to cause lethality in the next generation with only very limited persistence to subsequent generations.

Genetic control methods may be classified according to the expected persistence of the genetic element(s). For self-limiting systems, the modification is expected to disappear more or less rapidly from the environment if not maintained by release of additional modified insects. Conversely, in 'self-sustaining' systems the modification is expected to persist indefinitely without the

need for further releases, and in some cases to increase in frequency and/or invade adjacent populations (reviewed by Hay *et al.*, 2021). Self-limiting systems are, by their nature, more controllable and reversible than self-sustaining systems (Braig and Yan, 2001; James, 2005) and therefore seen as lower risk and more appropriate for initial use, at least in the context of transgenic strains (FAO/IAEA, 2002; Benedict and Robinson, 2003; Ågren and Clark, 2018).

Sterile insect methods, both classical SIT and its genetic descendants and cousins, are clearly genetic control systems, with the genetic element – radiation-induced lethal mutation, transgene or whatever – impacting on the wild population through mating. Furthermore, these are all strongly self-limiting systems, as the fitness penalty associated with the lethal or sterile trait means that it will disappear rapidly from the population if not maintained by periodic release of additional modified insects.

Moreover, these systems share common advantages, for example being highly target-specific in effect – meaning minimal impact on non-target species – and, in harnessing the mate-seeking instincts of male insects, capable of reaching pest insects that may be difficult to reach by other means.

23.3 Engineered Traits

How then might the SIT be improved through genetics? The main focus of this chapter is on the use of conditional dominant lethal genes, which can be used for sterilization and/or sex separation. When these conditional lethal genes are transgenes, i.e., constructed using recombinant DNA methods with at least some exogenous DNA, they are invariably associated with heritable markers. These have uses in their own right, and thereby add value to the use of transgenic methods, but are not further discussed here.

23.3.1 An alternative to sterilization by irradiation

Radiation generates dominant lethal mutations in the sperm of males; if such sperm

fertilizes eggs, these mutations lead to death of the zygote. Radiation therefore acts as a conditional (inducible) lethal genetic system. We may imagine replacing this with alternative conditional lethal systems to achieve similar effects, or subtly different effects that may have specific advantages. Sterilization by radiation has some disadvantages, both in terms of the process requirements and because the whole-body radiation used damages somatic as well as germline cells, and indeed also associated microbes such as gut flora (Ben Ami *et al.*, 2010; Lauzon and Potter, 2012). Both the logistical and the biological effects tend to increase the cost and decrease the effectiveness of the SIT.

Though radiation is presented above as an inducible lethal system, the term is more often applied to a conditional lethal that is present in a modified strain as a stable heritable trait, but only active under specific conditions. These might be normally absent and provided to give the effect (an inducible lethal system) or normally absent (a repressible lethal system). Efforts to date have largely focused on repressible lethal systems, where the lethal would be active except that it is repressed by providing a specific condition. The engineered strain then carries, typically in a homozygous state, a dominant lethal gene. By providing the repressor condition or 'antidote' – in several extant examples this is polyketide tetracycline, which can readily be provided in the mass-rearing diet – the strain may be propagated and amplified indefinitely. However, if males of such a strain are released into the environment they will seek out and mate with wild females. The progeny of such mating will inherit one copy of the lethal transgene and so, developing in the absence of the repressor condition, they will die. In this way the inheritance pattern and function of radiation-sterilized insects can be replicated, but without the need for radiation. This system was first developed to confer a bi-sex self-limiting trait (Thomas *et al.*, 2000), but a variant has been developed in which the lethal effect is female-specific, so that only female offspring die. These systems are illustrated diagrammatically in Fig. 23.1.
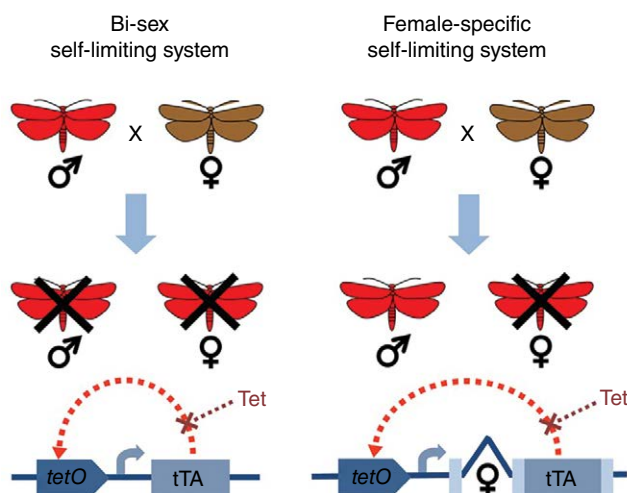
Fig. 23.1. Schematic diagrams illustrating two variants of the self-limiting technology. In the bi-sex self-limiting system (left), insects of both sexes die in the absence of a dietary repressor (tetracycline or suitable analogues). The genetic circuit underpinning this, illustrated diagrammatically below, is based on a positive-feedback system comprising two interacting genetic components: the tetracycline response element, *tetO*, and the tetracycline-repressible transcription factor, tTA. In the absence of tetracycline the resulting very high levels of tTA production cause mortality; in the presence of tetracycline the positive feedback loop is blocked, as tetracycline-bound tTA does not bind DNA (Gossen and Bujard, 1992; Gossen *et al*., 1994). In the female-specific self-limiting system (right), tTA expression is further regulated by inserting its coding sequence into, or adjacent to, a sequence that shows alternative splicing in males and females (female-specific intron indicated). If the female splice variant encodes functional tTA but the male equivalent does not, the lethal positive feedback system will be female-specific. This outcome allows for male-only production on a mass-rearing scale, and results in death of female progeny in the field.

A repressible lethal system has a 'fail-safe' or 'biocontainment' aspect that is missing from inducible systems (Alphey, 2002; Alphey and Andreasen, 2002). If the strain is inherently sterile or inviable and maintained in culture only by provision of an antidote to the lethal system, it cannot establish in the wild, even if the sterility or lethality is not 100% penetrant. In contrast, with an inducible system such as radiation, viable fertile pest insects are mass-reared and only become control agents if the sterilizing system or inducer is correctly applied. In fact, the SIT has a very good safety record in this regard, with (to date) no mass escapes from mass-rearing facilities; however, some technical failures have led to the release of fertile insects (del Valle, 2003). The response to these inadvertent releases of fertile insects was to release more sterile insects to deal with them, illustrating confidence in the technology. Even modest numbers of fertile flies escaping from a rearing facility may be problematic for an elimination programme.

23.3.2 Genetic sexing

It is generally preferred that the released sterile insects should all be male. In some cases, sterile females may be harmful. This is the case for mosquitoes, where females bite but males do not, and for tephritid fruit flies where female oviposition damages the fruit irrespective of whether the egg hatches to a larva. More fundamentally, released sterile females probably contribute little to population suppression, as the capacity of the males to fertilize females is rarely limiting for population dynamics. Instead, they may 'distract' co-released sterile males from seeking out wild females and thereby actually reduce the potential effect of the released

sterile males on the wild population (Zervas and Economopoulos, 1982; Rendón *et al*., 2004). In large field trials with irradiated medfly, male-only releases were found to be from three- to fivefold more effective per male than mixed-sex releases (Rendón *et al*., 2004); this large factor would justify considerable effort to develop sex-separation methods.

While sex separation can be achieved for some species based on some natural sexual dimorphism, for example pupal size for *Aedes aegypti* (Fay and Morlan, 1959; Ansari *et al*., 1977; Focks, 1980), this is not the case for most species of interest and manual sex sorting reduces programmatic efficiency. Sex separation can be induced by genetics, to give a genetic sexing strain (GSS). Indeed, the medfly experiment of Rendón *et al*. (2004) described above used such a strain, in which heat treatment of embryos gave a substantially male-only population based on a temperature-sensitive lethal. These strains were developed by the International Atomic Energy Agency using classical genetics (Robinson *et al*., 1999); similar translocation-based sexing strains were also developed for some mosquito species, using insecticide resistance as the selectable marker (Dame *et al*., 2009; Yamada *et al*., 2012). However, the mutants and special chromosomes required for such strains cannot be transferred between sexually incompatible species, therefore such strains must be developed independently each time. Transgenic methods, in contrast, may allow reuse of constructs and designs between species, allowing more rapid, systematic and predictable development of such strains (Alphey *et al*., 2008; Papathanos *et al*., 2009). This prospect seems to have been borne out in practice, with individual constructs working across a range of higher Diptera (Fu *et al*., 2007; Ant *et al*., 2012), mosquitoes (Fu *et al*., 2007; Labbé *et al*., 2012; Marinotti *et al*., 2013) or moths (Jin *et al*., 2013; Tan *et al*., 2013) (see Scott *et al*., Chapter 17, this volume). In other cases, generic molecular designs have worked across species if particular species-specific components are replaced (Schetelig and Handler, 2012b; Ogaugwu *et al*., 2013) (see Handler and Schetelig, Chapter 21, this volume).

23.3.3 Combining genetic sexing and population suppression

The transgenic sexing strains (sometimes called TSS to distinguish from GSS made using classical genetics) are based on the use of conditional female-specific dominant lethals. For a TSS there is no particular need for the lethal effect to be dominant, as it will likely be present in two copies in the strain at the point of sex separation. In all of the above examples the lethality is repressible; this again is not a requirement for a sexing strain, but it does allow the TSS construct also to serve as a 'sterilization' system. In this female-specific self-limiting approach, the female-specific lethal is used twice, for slightly different purposes.

The first is genetic sexing. The strain, homozygous for a repressible female-specific dominant lethal, is normally reared in the presence of the 'antidote' or repressor condition. The cohorts intended for release are reared without the antidote; females die due to the action of the female-specific lethal, giving a male-only cohort for release. This is genetic sexing.

After release, these males, still homozygous for a repressible female-specific dominant lethal, mate with wild females. Offspring from such a cross inherit one copy of the female-specific dominant lethal, which leads to the death of female offspring. This is akin to 'genetic sterilization'. However, in contrast to conventional SIT, male offspring survive; these are heterozygous for the female-specific dominant lethal and half of their female offspring die. This female-specific self-limiting method is more closely related to the field female-killing (FFK) developed in *Lucilia cuprina* (Whitten and Foster, 1975; Foster *et al*., 1988) than to classical SIT (Black *et al*., 2011), though still within the definition of 'genetic sterilization' in Box 23.1. Though the transgene will persist in the target population somewhat longer than a fully penetrant bi-sex lethal, due to the survival of heterozygous males, it will still be rapidly eliminated if not maintained by additional releases, due to the large fitness penalty associated with female-specific lethality. This is therefore a self-limiting

genetic control method, as with other sterile-insect methods. Modelling indicates that releases of males carrying female-specific self-limiting genes can be considerably more effective than systems in which both sexes of offspring are killed, such as conventional SIT, especially if strains carrying the transgene at multiple unlinked loci are used (Schliekelman and Gould, 2000; Thomas *et al*., 2000; Gould and Schliekelman, 2004). Contained glasshouse studies conducted with strains of medfly, olive fly and diamondback moth, all carrying female-specific self-limiting genes, have demonstrated that releases of self-limiting males are effective in reducing target populations of wild-type counterparts to elimination within a few weeks (Ant *et al*., 2012; Leftwich *et al*., 2014; Harvey-Samuel *et al*., 2015). Releases of males of a similar strain of the mosquito *Ae. aegypti* have also been highly successful in reducing populations of the mosquito in urban communities in Brazil (described further in section 23.11).

23.4   Integrated Pest Management

Sterile-male methods can work as stand-alone population control methods, but are better used as part of an integrated pest management system (IPM) (for disease vectors this is often called integrated vector management, IVM). While it is obvious in general that optimal use of multiple methods will give better results than use of only a single method, there are some specific features of sterile-insect methods that make them likely to be useful in an IPM context. One of these is the use of sterile-insect methods, particularly female-specific self-limiting insects, for resistance management (see section 23.5, below). Another is that sterile-insect methods are more effective when the ratio of sterile to fertile males is high, as more wild females will then mate a sterile male than if the ratio is lower. If a control programme releases sterile males at a constant rate, as the target population starts to decline, this sterile:wild male ratio will increase. Consequently, the programme will

become progressively more effective. This is extremely unusual – most pest control methods can achieve some degree of suppression but then become increasingly *less* effective as the density of the target diminishes, or where the remaining population 'hides' in areas that the operator or control agent (e.g., chemical) does not reach. This feature makes sterile-insect methods exceptionally powerful where suppression to near-zero levels, or actual elimination, is required. In general, it suggests that the optimal mix in many cases may be initial suppression using conventional means, followed by use of sterile insects to target the now-reduced wild population, but still in combination with other approaches for optimum efficacy and resistance management. Operational SIT programmes indeed typically use IPM principles (Bloem *et al*., 2005; Hendrichs *et al*., 2005, 2007; Henneberry, 2007; Grefenstette *et al*., 2009).

23.5   Resistance Management

Use of multiple pest control methods with independent resistance mechanisms, i.e., no cross-resistance, can reduce the selective advantage of any one resistance allele and therefore tends to reduce or prevent its spread in the target pest population. As any potential resistance to other control methods, such as chemical, biological or plant-incorporated toxins, is unlikely to confer cross-resistance to sterile-insect methods (and vice versa), use of sterile-insect methods within an IPM programme can provide a degree of resistance management. This has been a key element of the pink bollworm eradication programme in the south-western USA (Tabashnik *et al*., 2010, 2012).

Other sterile-insect variants may be more effective for resistance management. In conventional SIT, if fully sterile insects are used there is no introgression of background wild-type genes from the mass-reared population into the wild population. However, in the female-specific self-limiting approach, the survival of heterozygous males means that there is such introgression. If the mass-reared strain is susceptible to

another relevant control agent, for example *Bt* or a chemical insecticide, then this ongoing introgression of susceptible alleles from the mass-reared population into the wild population will tend to counter the spread of any potential resistance allele that may be present or arise in the target population. Modelling indicates that this can provide an extremely powerful resistance management tool, with the potential to work in complement with, and protect the effectiveness of, insecticides and *Bt*-expressing crops (Alphey *et al.*, 2007, 2009). In more recent empirical studies, releases of male diamondback moths carrying a female-specific self-limiting gene slowed the spread of resistance to *Bt*-expressing broccoli in glasshouse cage populations of the pest (Harvey-Samuel *et al.,* 2015). Development is under way with a similar strain of fall armyworm as a pest and resistance management tool (Oxitec, 2021a). An effective resistance management approach is particularly needed in biotech corn in Brazil, where resistance management is currently inadequate, meaning that fall armyworm rapidly develops resistance to new *Bt* traits (Fatoretto *et al.*, 2017).

23.6   Molecular Designs

At present a relatively small number of molecular designs have been used for transgene-based conditional lethals in pest insects. Extant systems are almost all based on the 'Tet-Off' tetracycline-repressible gene expression system (Gossen and Bujard, 1992; Gossen *et al.*, 1994) (see Schetelig *et al.*, Chapter 2, this volume), though Christophides *et al.* (2001) provided an example of a different design. Tet-Off systems may be in the conventional 'bipartite' configuration of promoter-tTA with tRE-effector (Bello *et al.*, 1998; Heinrich and Scott, 2000; Thomas *et al.*, 2000) or a one-part 'positive feedback' configuration (Gong *et al.*, 2005; Koukidou *et al.*, 2006; Phuc *et al.*, 2007; Morrison *et al.*, 2012). This latter arrangement removes the need for a specific promoter and has allowed use in a wide species range, but at the cost of losing the tissue- and developmental stage-specificity of expression that a suitable promoter may confer. Embryonic promoters, for example, have been used to give embryonic lethality (Horn and Wimmer, 2003; Schetelig *et al.*, 2009; Schetelig and Handler, 2012a), which has not generally been observed in positive feedback designs.

Female-specific expression has been achieved using female-specific promoters (Heinrich and Scott, 2000; Thomas *et al.*, 2000; Fu *et al.*, 2010) or of sex-specific alternative splicing cassettes based on *transformer* (Fu *et al.*, 2007; Ant *et al.*, 2012; Concha *et al.*, 2016; Schetelig *et al.*, 2021) or *doublesex* homologues (Jin *et al.*, 2013; Tan *et al.*, 2013). These alternative splicing systems can function early in development (Schetelig and Handler, 2012b) when sex-specific promoters may be difficult to obtain. They also potentially allow combinatorial control of gene expression, with expression depending on the combination of promoter and splicing activities.

23.7   Choosing an Effector

Considering that almost any protein is likely to disrupt cellular functions if overexpressed at a sufficiently high level, it is perhaps surprising that only a relatively small number of lethal effector molecules have been published so far. These include pro-apoptotic proteins or mutant derivatives (Heinrich and Scott, 2000; Horn and Wimmer, 2003; Fu *et al.*, 2010), tTA or VP16 (Gong *et al.*, 2005; Phuc *et al.*, 2007; Fu *et al.*, 2010; Jin *et al.*, 2013), *Ras64B*[V12] (Thomas *et al.*, 2000) and the type 1 serine/threonine protein phosphatase inhibitor NIPP1Dm (Parker *et al.*, 2002; Bennett *et al.*, 2003; Fu *et al.*, 2010). Presumably many more effectors could be used, including functional RNAs as well as proteins. That such a wide range of modes of action is available is encouraging in terms of managing resistance (see below).

Mode of action has been and continues to be an important consideration in the regulation of pesticidal chemicals, where it is

predominantly required to assess potential chemical metabolites and their safety to human health and the environment. In biological systems rather different concerns and issues apply, so the focus is on analysing the novel trait and other potential effects on the insect, and the implications of these for human health and the environment, using a 'weight of evidence' approach. Transgenic effectors have been deliberately selected on the basis of some knowledge-based prediction of their likely effect and mode of action. Consequently, how these effectors work is very clear, as it is through overexpression of this single, well-defined molecule. Effectors with multiple targets, such as tTA/VP16, which is thought to act via transcriptional squelching (Gill and Ptashne, 1988; Shockett *et al*., 1995; Baron *et al*., 1997), are preferred, to minimize the possibility of target-site resistance arising. An extreme example of multiple targets is radiation sterilization, where the random nature of the induced dominant lethal mutations means that the killing mechanism will be different from one individual to another.

23.8 Choice of Switch

Radiation and *Wolbachia*-induced cytoplasmic incompatibility (CI) represent forms of conditional lethality. For transgenes where a conditional switch has been used it has been the tetracycline-responsive Tet-Off system (Gossen and Bujard, 1992) or occasionally the related Tet-On (Gossen *et al*., 1994). Other switches have been used in *Drosophila melanogaster*, especially temperature (e.g., McGuire *et al*., 2003; Zeidler *et al*., 2004), but also chemical switches (Osterwalder *et al*., 2001; Markaki *et al*., 2004). The Tet-Off system has considerable advantages. It is extremely well characterized, with over 7000 publications in peer-reviewed journals describing successful use in tissue culture systems, yeasts, protozoa, insects, plants and vertebrates, especially mice (TetSystems, 2010). The chemical switch, tetracycline, is itself well characterized due to its long use as an antibiotic, including for human therapy. In

this context it should be noted that these tetracycline-regulated systems are based on the tetracycline sensor *tet*R, not a resistance factor. A limitation with temperature, for example, as the basis of a conditional system is that temperature in the field is uncontrollable and may be quite variable. This raises the question of whether tetracycline might be found in the environment at sufficiently high concentrations to repress intended lethality. For this to be a possibility, sufficient tetracycline would have to be available to adults or developing larvae (depending on the precise system configuration) and therefore present in their specific habitats. These vary considerably by species and, as for any environmental risk assessment, this issue needs to be considered on a case-by-case basis. None the less, it may be useful to consider specific scenarios.

23.9 Strain Performance

Radiation sterilization has a negative impact on insects (e.g., Barry *et al*., 2003; Kraaijeveld and Chapman, 2004). An early concern was that genetic engineering might itself have a negative impact, reducing the benefit of replacing radiation with genetics. Early studies seemed to indicate this (Catteruccia *et al*., 2003; Irvin *et al*., 2004), but more recent analysis has shown that transgenic strains can be developed with acceptable performance in the assays used (Allen *et al*., 2004; Marrelli *et al*., 2006, 2007; Bargielowski *et al*., 2011a,b, 2012). In particular, various strains have performed well in tests of male mating competitiveness, which is a key performance parameter for sterile-male methods (Morrison *et al*., 2009; Schetelig *et al*., 2009; Ant *et al*., 2012; Jin *et al*., 2013; Lee *et al*., 2013). In the field, a transgenic strain of pink bollworm showed comparable performance to wild-type counterparts (Simmons *et al*., 2011).

23.10 Resistance

All interventions can provoke compensating genetic changes, which may manifest as

resistance to the intervention. This applies also to genetic methods, including sterile-insect methods. As well as biochemical resistance to the lethality or sterility mechanism there is the possibility of behavioural resistance, perhaps via selection for females rejecting sterile males, leading to assortative mating. In fact, SIT has a good record in this regard: assortative mating has rarely been observed in practice (Koyama *et al.*, 2004). The random nature of radiation-induced mutations may make biochemical resistance relatively difficult; this does not necessarily apply to other sterility systems, though female-lethal systems may inhibit the spread of resistance alleles in the wild population by introgressing susceptible alleles from the factory population (Alphey *et al.*, 2011).

23.11 Field Experience and Future Prospects

With over a decade of field experience of multiple successful field demonstrations with self-limiting insects, and two such strains approved for commercial biosafety in Brazil, the maturity of this approach has now largely shifted from early-stage research and proof-of-concept studies to one on the brink of delivering major impact against major threats to human health and food crops.

For mosquitoes, multiple field releases have been conducted with self-limiting *Ae. aegypti*, with multiple demonstrations of the effectiveness of such strains for substantial suppressive impact on treated field mosquito populations. Field releases of adult males of a bi-sex self-limiting strain, called OX513A, in Grand Cayman in 2009–2010 demonstrated good male mating competitiveness and significant suppression of the resident *Ae. aegypti* population (Harris *et al.*, 2011, 2012). Later field releases in Panama and Brazil underlined the high levels of vector management achievable with this strain, reaching 93–95% suppression of treated *Ae. aegypti* populations relative to those in untreated sites (Carvalho *et al.*, 2015; Gorman *et al.*, 2016). In the city of Piracicaba, Brazil,

programmatic deployment of OX513A culminated in treatment of communities numbering 65,000 people and was reported to achieve similar levels of *Ae. aegypti* control (unpublished data).

Later deployments have been conducted with a male-selecting self-limiting strain of *Ae. aegypti*, OX5034, which is designed to deliver new advantages relative to OX513A. Genetic sexing engineered in OX5034 bypasses the need for manual sex sorting, reducing costs and operational complexity. Releases of OX5034 males in the Brazilian city of Indaiatuba over two seasons have achieved > 90% suppression of treated *Ae. aegypti* populations (Oxitec, 2021 b,c). Brazilian government regulators have now approved OX5034 as safe for deployment nationwide (Barroso, 2020).

The male-selecting trait in OX5034 has also enabled a transition from releases of the fragile adult stage to deployment of the egg stage, offering greater shelf-life (*Ae. aegypti* eggs can be stored for multiple months) and robustness in transportation. At the time of writing, this egg deployment approach is being tested in regulated pilot field releases in the Florida Keys, USA.

For crop pests, strains of pink bollworm, diamondback moth, tephritid fruit flies and *Drosophila suzukii* have shown strong mating performance, suppressed pest populations and demonstrated insecticide resistance management in cage trials (Morrison *et al.*, 2009; Schetelig *et al.*, 2009; Ant *et al.*, 2012; Leftwich *et al.*, 2014; Harvey-Samuel *et al.*, 2015). The first genetically engineered insect used in field trials was a strain of pink bollworm expressing the fluorescent protein DsRed2 to provide a heritable marker (Simmons *et al.*, 2011) (see Scott *et al.*, Chapter 17, this volume); these moths were sterilized by irradiation. From 2006, trials over several years involving the release of > 15 million GE moths showed that the strain was competitive and the marker trait stable and effective (Simmons *et al.*, 2011; Walters *et al.*, 2012). Subsequently, field releases of a male-selecting self-limiting strain of diamondback moth, in upstate New York, USA, demonstrated that such strains can perform strongly in the

field (Shelton *et al*., 2020). These results are highly encouraging for the future implementation of such strains in agriculture, and recent biosafety approval of a male-selecting, self-limiting fall armyworm indicates that the first commercial application of self-limiting insects is imminent (Barroso, 2021).

We are now realizing the potential of biotechnology to broaden the application of SIT-like methods beyond the notable successes achieved with the conventional SIT to date. The self-limiting approach is now also evolving, with development under way in new target pests and the focus also shifting to optimizing the technology and processes to deliver greater operational and cost efficiencies for maximum future impact and accessibility. Other pest management tools are under pressure in the face of pest resistance (Tabashnik *et al*., 2013; Sparks and Nauen, 2015; Ranson and Lissenden, 2016; Gould *et al*., 2018), driving greater demand for novel additions to integrated pest management options, to reduce over-reliance on single methods and, as we have described in this chapter, even counteract development of resistance in pests. Climate change and increasingly invasive pests will exacerbate these challenges (Paini *et al*., 2016; Halsch *et al*., 2021). Over the coming decade, as this field continues to expand and develop in a world that increasingly demands environmental sustainability, self-limiting insects are expected to become established as an important mainstream pest suppression and resistance management tool for public health and agriculture.

Acknowledgements

References

Ågren, J.A. and Clark, A.G. (2018) Selfish genetic elements. *PLoS Genetics* 14, e1007700.

Allen, M., Berkebile, D. and Skoda, S. (2004) Postlarval fitness of transgenic strains of *Cochliomyia hominivorax* (Diptera: Calliphoridae). *Journal of Economic Entomology* 97, 1181–1185.

Alphey, L. (2002) Re-engineering the Sterile Insect Technique. *Insect Biochemistry & Molecular Biology* 32, 1243–1247.

Alphey, L. and Andreasen, M.H. (2002) Dominant lethality and insect population control. *Molecular Biochemistry & Parasitology* 121, 173–178.

Alphey, N., Coleman, P.G., Donnelly, C.A. and Alphey, L. (2007) Managing insecticide resistance by mass release of engineered insects. *Journal of Economic Entomology* 100, 1642–1649.

Alphey, L., Nimmo, D., O'Connell, S. and Alphey, N. (2008) Insect population suppression using engineered insects. In: Aksoy, S. (ed.) *Transgenesis and the Management of Vector-Borne Disease*. Landes Bioscience, Austin, Texas, pp. 93–103.

Alphey, N., Bonsall, M. and Alphey, L. (2009) Combining pest control and resistance management: synergy of engineered insects with *Bt* crops. *Journal of Economic Entomology* 102, 717–732.

Alphey, L., Benedict, R., Bellini, R., Clark, G.G., Dame, D.A. *et al.* (2010) Sterile-insect methods for control of mosquito-borne diseases: an analysis. *Vector Borne & Zoonotic Diseases* 10, 295–311.

Alphey, N., Bonsall, M.B. and Alphey, L. (2011) Modeling resistance to genetic control of insects. *Journal of Theoretical Biology* 270, 42–55.

Ansari, M., Singh, K., Brooks, G., Malhotra, P. and Vaidyanathan, V. (1977) The development of procedures and techniques for mass rearing of *Aedes aegypti*. *Indian Journal of Medical Research* 65 (Suppl.), 91–99.

Ant, T., Koukidou, M., Rempoulakis, P., Gong, H.-F., Economopoulos, A. *et al.* (2012) Control of the olive fruit fly using genetics-enhanced sterile insect technique. *BMC Biology* 10, 51.

Bargielowski, I., Alphey, L. and Koella, J.C. (2011a) Cost of mating and insemination capacity of a genetically modified mosquito *Aedes aegypti* OX513A compared to its wild type counterpart. *PLoS ONE* 6, e26086.

Bargielowski, I., Nimmo, D., Alphey, L. and Koella, J.C. (2011b) Comparison of life history characteristics of the genetically modified OX513A line and a wild type strain of *Aedes aegypti*. *PLoS ONE* 6, e20699.

Bargielowski, I., Kaufmann, C., Alphey, L., Reiter, P. and Koella, J. (2012) Flight performance and teneral energy reserves of two genetically modified and one wild type strain of the yellow fever mosquito, *Aedes aegypti* (Diptera: Culicidae). *Vector Borne and Zoonotic Diseases* 12, 1053–1058.

Baron, U., Gossen, M. and Bujard, H. (1997) Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential. *Nucleic Acids Research* 25, 2723–2729.

Barroso, P.A. (2020) Technical opinion for the commercial release of the genetically modified mosquito *Aedes aegypti* strain OX5034. Document 6946/2020. National Technical Commission on Biosafety (CTNBio), Brasilia, Brazil.

Barroso, P.A. (2021) Technical opinion for the commercial release of moth *Spodoptera frugiperda* genetically modified strain OX5382G. Document 7350/2021. National Technical Commission on Biosafety (CTNBio), Brasilia, Brazil.

Barry, J., McInnis, D., Gates, D. and Morse, J. (2003) Effects of irradiation on Mediterranean fruit flies (Diptera: Tephritidae): emergence, survivorship, lure attraction, and mating competition. *Journal of Economic Entomology* 96, 615–622.

Bello, B., Resendez-Perez, D. and Gehring, W. (1998) Spatial and temporal targeting of gene expression in *Drosophila* by means of a tetracycline-dependent transactivator system. *Development* 125, 2193–2202.

Ben Ami, E., Yuval, B. and Jurkevitch, E. (2010) Manipulation of the microbiota of mass-reared Mediterranean fruit flies *Ceratitis capitata* (Diptera: Tephritidae) improves male sexual performance. *International Society for Microbial Ecology* 4, 28–37.

Benedict, M.Q. and Robinson, A.S. (2003) The first releases of transgenic mosquitoes: an argument for the sterile insect technique. *Trends in Parasitology* 19, 349–355.

Bennett, D., Szöor, B., Gross, S., Vereshchagina, N. and Alphey, L. (2003) Ectopic expression of inhibitors of protein phosphatase type 1 (PP1) can be used to analyze roles of PP1 in *Drosophila* development. *Genetics* 164, 235–245.

Black, W.C. IV, Alphey, L. and James, A.A. (2011) Why RIDL is not SIT. *Trends in Parasitology* 27, 362–370.

Bloem, K.A., Bloem, S. and Carpenter, J.E. (2005) Impact of moth suppression/eradication programmes using the sterile insect technique or inherited sterility. In: Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) *Sterile Insect Technique Principles and Practice in Area-wide Integrated Pest Management*. Springer, Dordrecht, Netherlands, pp. 677–700.

Braig, H. and Yan, G. (2001) The spread of genetic constructs in natural insect populations. In: Letourneau, D.K. and Burrows, B.E. (eds) *Genetically Engineered Organisms: Assessing Environmental and Human Health Effects*. CRC Press, Boca Raton, Florida.

Breeland, S.G., Jeffery, G.M., Lofgren, C.S. and Weidhaas, D.E. (1974) Release of chemosterilized males for the control of *Anopheles albimanus* in El Salvador. I. Characteristics of the test site and the natural population. *The American Journal of Tropical Medicine and Hygiene* 23, 274–280.

Carvalho, D.O., McKemey, A.R., Garziera, L., Lacroix, R., Donnelly, C.A. *et al.* (2015) Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. *PLoS Neglected Tropical Diseases* 9, e0003864.

Catteruccia, F., Godfray, H.C. and Crisanti, A. (2003) Impact of genetic manipulation on the fitness of *Anopheles stephensi* mosquitoes. *Science* 299, 1225–1227.

Chambers, E.W., Hapairi, L., Peel, B., Bossin, H. and Dobson, S. (2011) Male mating competitiveness of a *Wolbachia*-introgressed *Aedes polynesiensis* strain under semi-field conditions. *PLoS Neglected Tropical Diseases* 5, e1271.

Christophides, G., Savakis, C., Mintzas, A. and Komitopoulou, K. (2001) Expression and function of the *Drosophila melanogaster* ADH in male *Ceratitis capitata* adults: a potential strategy for medfly sexing based on gene-transfer technology. *Insect Molecular Biology* 10, 249–254.

Concha, C., Palavesam, A., Guerrero, F.D., Sagel, A., Li, F. *et al.* (2016) A transgenic male-only strain of the New World screwworm for an improved control program using the sterile insect technique. *BMC Biology* 14, 72.

Dame, D., Curtis, C., Benedict, M., Robinson, A. and Knols, B. (2009) Historical applications of induced sterilisation in field populations of mosquitoes. *Malaria Journal* 8, S2.

Davidson, G., Odetoyinbo, J.A., Colussa, B. and Coz, J. (1970) Field attempt to assess the mating competitiveness of sterile males produced by crossing 2 member species of the *Anopheles gambiae* complex. *Bulletin of the World Health Organization* 42, 55–67.

del Valle, J. (2003) New World screwworm (*Cochliomyia hominivorax*) in Mexico. *Disease Information* 16, 49–50.

Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) (2005) *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management*. Springer, Dordrecht, Netherlands.

FAO/IAEA (2002) *Status and Risk Assessment of the Use of Transgenic Arthropods in Plant Protection*. FAO/IAEA, Vienna, p. 48.

Fatoretto, J.C., Michel, A.P., Silva Filho, M.C. and Silva, N, (2017) Adaptive potential of fall armyworm (Lepidoptera: Noctuidae) limits *Bt* trait durability in Brazil. *Journal of Integrated Pest Management* 8, 1.

Fay, R. and Morlan, H. (1959) A mechanical device for separating the developmental stages, sexes and species of mosquitoes. *Mosquito News* 19, 144–147.

Focks, D.A. (1980) An improved separator for separating the developmental stages, sexes and species of mosquitoes. *Journal of Medical Entomology* 17, 567–568.

Foster, G., Vogt, W., Woodburn, T. and Smith, P. (1988) Computer simulation of genetic control. Comparison of sterile males and field-female killing systems. *Theoretical and Applied Genetics* 76, 870–879.

Fu, G., Condon, K.C., Epton, M.J., Gong, P., Jin, L. *et al.* (2007) Female-specific insect lethality engineered using alternative splicing. *Nature Biotechnology* 25, 353–357.

Fu, G., Lees, R.S., Nimmo, D., Aw, D., Jin, L. *et al.* (2010) Female-specific flightless phenotype for mosquito control. *Proceedings of the National Academy of Sciences USA* 107, 4550–4554.

Gill, G. and Ptashne, M. (1988) Negative effect of the transcriptional activator GAL4. *Nature* 334, 721–724.

Gong, P., Epton, M., Fu, G., Scaife, S., Hiscox, A. *et al.* (2005) A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nature Biotechnology* 23, 453–456.

Gorman, K., Young, J., Pineda, L., Márquez, R., Sosa, N. *et al.* (2016) Short-term suppression of *Aedes aegypti* using genetic control does not facilitate *Aedes albopictus*. *Pest Management Science* 72, 618–628.

Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences USA* 89, 5547–5551.

Gossen, M., Bonin, A., Freundlieb, S. and Bujard, H. (1994) Inducible gene expression systems for higher eukaryotic cells. *Current Opinion in Biotechnology* 5, 516–520.

Gould, F. and Schliekelman, P. (2004) Population genetics of autocidal control and strain replacement. *Annual Review of Entomology* 49, 193–217.

Gould, F., Brown, Z.S. and Kuzma, J. (2018) Wicked evolution: can we address the sociobiological dilemma of pesticide resistance? *Science* 360(6390), 728–732. doi: 10.1126/science.aar3780

Grefenstette, B., El-Lissy, O. and Staten, R.T. (2009) Pink Bollworm Eradication Plan in the U.S. *USDA-APHIS*. Available at: http://www.aphis.usda.gov/plant_health/plant_pest_info/cotton_pests/downloads/pbw-erad-plan2-09.pdf (accessed 4 January 2013)

Halsch, C.A., Shapiro, A.M., Fordyce, J.A., Nice, C.C., Thorne, J.H. *et al.* (2021) Insects and recent climate change. *Proceedings of the National Academy of Sciences, USA* 118, e2002543117.

Harris, A.F., Nimmo, D., McKemey, A.R., Kelly, N., Scaife, S. *et al.* (2011) Field performance of engineered male mosquitoes. *Nature Biotechnology* 29, 1034–1037.

Harris, A.F., McKemey, A.R., Nimmo, D., Curtis, Z., Black, I. *et al.* (2012) Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nature Biotechnology* 30, 828–830.

Harvey-Samuel, T., Morrison, N.I., Walker, A.S., Marubbi, T., Yao, J. *et al.* (2015) Pest control and resistance management through releases of insects carrying a male-selecting transgene. *BMC Biology* 13, 49.

Hay, B.A., Oberhofer, G. and Guo, M. (2021) Engineering the composition and fate of wild populations with gene drive. *Annual Review of Entomology* 66, 407–434.

Heinrich, J. and Scott, M. (2000) A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. *Proceedings of the National Academy of Sciences USA* 97, 8229–8232.

Hendrichs, J., Vreysen, M.J.B., Enkerlin, W.R. and Cayol, J.P. (2005) Strategic options in using sterile insects for area-wide integrated pest management. In: Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) *Sterile Insect Technique Principles and Practice in Area-wide Integrated Pest Management*. Springer, Dordrecht, Netherlands, pp. 563–600.

Hendrichs, J., Kenmore, P., Robinson, A. and Vreysen, M. (2007) Area-wide integrated pest management (AW-IPM): principles, practice and prospects. In: Vreysen, M., Robinson, A. and Hendrichs, J. (eds) *Area-Wide Control of Insect Pests: From Research to Field Implementation*. Springer, Dordrecht, Netherlands, pp. 3–33.

Henneberry, T.J. (2007) Integrated systems for control of the pink bollworm *Pectinophora gossypiella* in cotton. In: Vreysen, M.J.B., Robinson, A.S. and Hendrichs, J. (eds) *Area-Wide Control of Insect Pests*. Springer, Dordrecht, Netherlands, pp. 567–579.

Horn, C. and Wimmer, E. (2003) A transgene-based, embryo-specific lethality system for insect pest management. *Nature Biotechnology* 21, 64–70.

Irvin, N., Hoddle, M.S., O'Brochta, D.A., Carey, B. and Atkinson, P.W. (2004) Assessing fitness costs for transgenic *Aedes aegypti* expressing the GFP marker and transposase genes. *Proceedings of the National Academy of Sciences USA* 101, 891–896.

James, A.A. (2005) Gene drive systems in mosquitoes: rules of the road. *Trends in Parasitology* 21, 64–67.

Jin, L., Walker, A.S., Fu, G., Harvey-Samuel, T., Dafa'alla, T. *et al.* (2013) Engineered female-specific lethality for control of pest Lepidoptera. *ACS Synthetic Biology* 2, 166.

Klassen, W. and Curtis, C.F. (2005) History of the sterile insect technique. In: Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) *Sterile Insect Technique Principles and Practice in Area-wide Integrated Pest Management*. Springer, Dordrecht, Netherlands, pp. 3–36.

Knipling, E. (1955) Possibilities of insect control or eradication through the use of sexually sterile males. *Journal of Economic Entomology* 48, 459–469.

Koukidou, M., Klinakis, A., Reboulakis, C., Zagoraiou, L., Tavernarakis, N. *et al.* (2006) Germ line transformation of the olive fly *Bactrocera oleae* using a versatile transgenesis marker. *Insect Molecular Biology* 15, 95–103.

Koyama, J., Kakinohana, H. and Miyatake, T. (2004) Eradication of the melon fly *Bactrocera cucurbitae* in Japan: importance of behaviour, ecology, genetics and evolution. *Annual Review of Entomology* 49, 331–349.

Kraaijeveld, K. and Chapman, T. (2004) Effects of male sterility on female remating in the Mediterranean fruitfly, *Ceratitis capitata*. *Proceedings of the Royal Society (London) B* 271, S209–S211.

Labbé, G.M.C., Scaife, S., Morgan, S.A., Curtis, Z.H. and Alphey, L. (2012) Female-specific flightless (fsRIDL) phenotype for control of *Aedes albopictus*. *PLoS Neglected Tropical Diseases* 6, e1724.

Lauzon, C.R. and Potter, S.E. (2012) Description of the irradiated and nonirradiated midgut of *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) and *Anastrepha ludens* Loew (Diptera: Tephritidae) used for sterile insect technique. *Journal of Pest Science* 85, 217–226.

Lee, H.L., Vasan, S., Ahmad, N., Idris, I., Hanum, N. *et al.* (2013) Mating compatibility and competitiveness of transgenic and wild type *Aedes aegypti* (L.) under contained semi-field conditions. *Transgenic Research* 22, 47–57.

Leftwich, P.T., Koukidou, M., Rempoulakis, P., Gong, H-F., Zacharopoulou, A. *et al.* (2014) Genetic elimination of field-cage populations of Mediterranean fruit flies. *Proceedings of the Royal Society (London) B* 281, 20141372.

Lindquist, D.A., Abusowa, M. and Hall, M.J. (1992) The New World screwworm fly in Libya: a review of its introduction and eradication. *Medical and Veterinary Entomology* 6, 2–8.

Marinotti, O., Jasinskiene, N., Scaife, S., Fu, G., Brown, D. *et al.* (2013) Development of a population suppression strain for a vector of human malaria, *Anopheles stephensi. Malaria Journal* 12, 142.

Markaki, M., Craig, R.K. and Savakis, C. (2004) Insect population control using female specific pro-drug activation. *Insect Biochemistry and Molecular Biology* 34, 131–137.

Marrelli, M.T., Moreira, C.K., Kelly, D., Alphey, L. and Jacobs-Lorena, M. (2006) Mosquito transgenesis: what is the fitness cost? *Trends in Parasitology* 22, 197–202.

Marrelli, M.T., Li, C., Rasgon, J.L. and Jacobs-Lorena, M. (2007) Transgenic malaria-resistant mosquitoes have a fitness advantage when feeding on *Plasmodium*-infected blood. *Proceedings of the National Academy of Sciences USA* 104, 5580–5583.

McGuire, S.E., Le, P.T., Osborn, A.J., Matsumoto, K. and Davis, R.L. (2003) Spatiotemporal rescue of memory dysfunction in *Drosophila*. *Science* 302, 1765–1768.

Morrison, N.I., Segura, D.F., Stainton, K.C., Fu, G., Donnelly, C.A. and Alphey, L.S. (2009) Sexual competitiveness of a transgenic sexing strain of the Mediterranean fruit fly, *Ceratitis capitata*. *Entomologia Experimentalis et Applicata* 133, 146–153.

Morrison, N.I., Simmons, G.S., Fu, G., O'Connell, S., Walker, A.S. *et al.* (2012) Engineered repressible lethality for controlling the pink bollworm, a lepidopteran pest of cotton. *PLoS ONE* 7, e50922.

Ogaugwu, C.E., Schetelig, M.F. and Wimmer, E.A. (2013) Transgenic sexing system for *Ceratitis capitata* (Diptera: Tephritidae) based on female-specific embryonic lethality. *Insect Biochemistry and Molecular Biology* 43, 1–8.

Osterwalder, T., Yoon, K., White, B. and Keshishian, H. (2001) A conditional tissue-specific transgene expression system using inducible GAL4. *Proceedings of the National Academy of Sciences USA* 98, 12596–12601.

Oxitec (2021a) *Friendly ™ Fall Armyworm*. *Challenges to food security require innovative solutions: Oxitec is developing next-generation technology to counter fall armyworm.* Available at: https://www.oxitec.com/en/friendly-faw (accessed 1 September 2021)

Oxitec (2021b) *Tet Technology*. Available at: https://tetsystems.com/science-technology (accessed 1 September 2021)

Oxitec (2021c) *Oxitec's friendly mosquito technology receives US EPA approval for pilot projects in US*. Available at: https://www.oxitec.com/en/news/oxitecs-friendly-mosquito-technology-receives-us-epa-approval-for-pilot-projects-in-us-c2943 (accessed 1 September 2021)

Paini, D.R., Sheppard, A.W., Cook, D.C., De Barro, P.J., Worner, S.P. and Thomas, M.B. (2016) Global threat to agriculture from invasive species. *Proceedings of the National Academy of Sciences, USA* 113, 7575–7579.

Papathanos, P., Bossin, H., Benedict, M., Catteruccia, F., Malcolm, C. *et al.* (2009) Sex separation strategies: past experience and new approaches. *Malaria Journal* 8, S5.

Parker, L., Gross, S., Beullens, M., Bollen, M., Bennett, D. and Alphey, L. (2002) Functional interaction between nuclear inhibitor of protein phosphatase type 1 (NIPP1) and protein phosphatase type 1 (PP1) in *Drosophila*: consequences of over-expression of NIPP1 in flies and suppression by co-expression of PP1. *Biochemical Journal* 368, 789–797.

Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J. *et al.* (2007) Late-acting dominant lethal genetic systems and mosquito control. *BMC Biology* 5, 11.

Ranson, H. and Lissenden, A. (2016) Insecticide resistance in African *Anopheles* mosquitoes: a worsening situation that needs urgent action to maintain malaria control. *Trends in Parasitology* 32, 187–196.

Rendón, P., McInnis, D., Lance, D. and Stewart, J. (2004) Medfly (Diptera: Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. *Journal of Economic Entomology* 97, 1547–1553.

Robinson, A., Franz, G. and Fisher, K. (1999) Genetic sexing strains in the medfly, *Ceratitis capitata*: development, mass rearing and field application. *Trends in Entomology* 2, 81–104.

Schetelig, M.F. and Handler, A.M. (2012a) Strategy for enhanced transgenic strain development for embryonic conditional lethality in *Anastrepha suspensa*. *Proceedings of the National Academy of Sciences USA* 109, 9348–9353.

Schetelig, M.F. and Handler, A.M. (2012b) A transgenic embryonic sexing system for *Anastrepha suspensa* (Diptera: Tephritidae). *Insect Biochemistry and Molecular Biology* 42, 790–795.

Schetelig, M., Caceres, C., Zacharopoulou, A., Franz, G. and Wimmer, E.A. (2009) Conditional embryonic lethality to improve the sterile insect technique in *Ceratitis capitata* (Diptera: Tephritidae). *BMC Biology* 7, 4.

Schetelig, M.F., Schwirz, J. and Yan, Y. (2021) A transgenic female killing system for the genetic control of *Drosophila suzukii*. *Scientific Reports* 11(1), 12938.

Schliekelman, P. and Gould, F. (2000) Pest control by the release of insects carrying a female-killing allele on multiple loci. *Journal of Economic Entomology* 93, 1566–1579.

Shelton, A.M., Long, S.J., Walker, A.S., Bolton, M., Collins, H.L. *et al.* (2020) First field release of a genetically engineered, self-limiting agricultural pest insect: evaluating its potential for future crop protection. *Frontiers in Bioengineering and Biotechnology* 29, 482.

Shockett, P., Difilippantonio, M., Hellman, N. and Schatz, D.G. (1995) A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proceedings of the National Academy of Sciences USA* 92, 6522–6526.

Simmons, G.S., McKemey, A.R., Morrison, N.I., O'Connell, S., Tabashnik, B.E. *et al.* (2011) Field performance of a genetically engineered strain of pink bollworm. *PLoS ONE* 6, e24110.

Sparks, T.C. and Nauen, R. (2015) IRAC: Mode of action classification and insecticide resistance management. *Pesticide Biochemistry and Physiology* 121, 122–128.

Suckling, D., Barrington, A., Chhagan, A., Stephens, A., Burnip, G. *et al.* (2007) Eradication of the Australian painted apple moth *Teia anartoides* in New Zealand: trapping, inherited sterility, and male competitiveness. In: Vreysen, M., Robinson, A. and Hendrichs, J. (eds) *Area-Wide Control of Insect Pests*. Springer, Dordrecht, Netherlands, pp. 603–615.

Tabashnik, B.E., Sisterson, M.S., Ellsworth, P.C., Dennehy, T.J., Antilla, L. *et al.* (2010) Suppressing resistance to *Bt* cotton with sterile insect releases. *Nature Biotechnology* 28, 1304–1307.

Tabashnik, B.E., Morin, S., Unnithan, G.C., Yelich, A.J., Ellers-Kirk, C. *et al.* (2012) Sustained susceptibility of pink bollworm to *Bt* cotton in the United States. *GM Crops and Food: Biotechnology in Agriculture and the Food Chain* 3, 194–200.

Tabashnik, B., Brévault, T. and Carrière, Y. (2013) Insect resistance to *Bt* crops: lessons from the first billion acres. *Nature Biotechnology* 31, 510–521.

Tan, A., Fu, G., Li, J., Guo, Q., Li, Z. *et al.* (2013) A transgene-based, female-specific lethality system for genetic sexing of the silkworm, *Bombyx mori*. *Proceedings of the National Academy of Sciences USA* 110, 6766–6770.

TetSystems (2010) *Science and Technology*. *Principles and components [of Tet technology]*. Available at: https://www.tetsystems.com/science-technology (accessed April 2022)

Thomas, D.D., Donnelly, C.A., Wood, R.J. and Alphey, L.S. (2000) Insect population control using a dominant, repressible, lethal genetic system. *Science* 287, 2474–2476.

Vanderplank, F.L. (1944) Hybridization between *Glossina* species and suggested new method for control of certain species of tsetse. *Nature* 154, 607–608.

Vargas, T., Hursey, B. and Cunningham, E. (1994) Eradication of the screwworm from Libya using the sterile insect technique. *Parasitology Today* 10, 199–122.

Walters, M., Morrison, N.I., Claus, J., Tang, G., Phillips, C.E. *et al.* (2012) Field longevity of a fluorescent protein marker in an engineered strain of the pink bollworm, *Pectinophora gossypiella* (Saunders). *PLoS ONE* 7, e38547.

Whitten, M. and Foster, G. (1975) Genetical methods of pest control. *Annual Review of Entomology* 20, 461–476.

Yamada, H., Benedict, M., Malcolm, C., Oliva, C., Soliban, S. and Gilles, J. (2012) Genetic sex separation of the malaria vector, *Anopheles arabiensis*, by exposing eggs to dieldrin. *Malaria Journal* 11, 208.

Zeidler, P., Tan, C., Bellaiche, Y., Cherry, S., Häder, S. *et al.* (2004) Temperature-sensitive control of protein activity by conditionally splicing inteins. *Nature Biotechnology* 22, 871–876.

Zervas, G. and Economopoulos, A. (1982) Mating frequency in caged populations of wild and artificially reared (normal or γ-sterilized) olive fruit flies. *Environmental Entomology* 11, 17–20.

24 Public Acceptability and Stakeholder Engagement for Genetic Control Technologies

Delphine Thizy[1]*, Lucy Carter[2], Isabelle Coche[3], Jason A. Delborne[4], Claudia Emerson[5], Ana Kormos[6], Aditi Mankad[2], Lea Pare Toe[7], Aaron Roberts[5] and Yasha Rohwer[8]

[1]*Delphine Thizy Consulting, Belgium;* [2]*Land and Water, CSIRO, Australia;* [3]*Emerging Ag, Canada;* [4]*Genetic Engineering and Society Center, North Carolina State University, USA;* [5]*Institute on Ethics and Policy for Innovation, McMaster University, Canada;* [6]*University of California Davis, University of California Irvine Malaria Initiative, USA;* [7]*Institut de Recherche en Sciences de la Santé, Burkina Faso;* [8]*Humanities and Social Sciences Department, Oregon Tech, USA*

24.1 Introduction

The development of genetic control technologies as potential tools to complement existing vector control interventions in public health, pest control interventions in agriculture and invasive species control for conservation has been accompanied by increased attention to the question of public acceptability. While the adoption of innovative tools is usually driven by consideration of their efficacy, safety and comparative advantage vis-à-vis existing tools (WHO, 2017a), the public's understanding, perception and potential acceptance of new tools play a critical role in their adoption.

The concept of public 'acceptability' for a technology can refer to public's perception prior to its use, as well as satisfaction and feedback once the technology is used (Nadal *et al.*, 2019). In this chapter, we refer to the notion of acceptability prior to its use – in other words, the public perception of transgenic insects while research is ongoing, both before and during field evaluations, and prior to its utilization as a public health, conservation or pest control intervention.

The acceptability of innovations such as genetic control technologies is influenced by many factors (Cisnetto and Barlow, 2020), including the perceived need for innovation, the benefits and costs of current methods of control, familiarity with genetic science, potential concerns about research agendas of actors coming from a different region, worldviews about human relationships with 'nature', and cultural beliefs and values. Given this complexity, and growing public demand for transparency and participation in decision making, the willingness of technology developers to engage early and in good faith with stakeholders and potentially affected or participating communities is an important factor in public acceptability.

*Corresponding author, email: delphine@thizyconsulting.com

Researchers and developers must be prepared to work closely with affected communities in a collaborative fashion and need to establish procedures that ensure outcomes are legitimate and fair and perceived as such by those affected communities. Similarly, researchers and developers need to be clear and explicit early on in the process with other stakeholders about the opportunities for dialogue and for their concerns, expectations and inputs to be heard and considered.

The National Academies of Sciences, Engineering and Medicine (NASEM) defines engagement as 'seeking and facilitating the sharing and exchange of knowledge, perspectives, and preferences between or among groups who often have differences in expertise, power, and values' (NASEM, 2016). Such work enables mutual learning among people with different sources and kinds of expertise, two-way communication that creates understanding about interests and values, and trust-building.

Engagement is considered not only as a process related to public acceptability of a technology: it is also first and foremost a means of ensuring the relevance of proposed research to the affected community (CIOMS, 2016). It can have other purposes, such as participating in knowledge co-creation with researchers (Hartley *et al.*, 2019), feeding back to the development process (Buchthal *et al.*, 2019) and participating in the risk assessment of the technology (EFSA *et al.*, 2020). However, for the purpose of this chapter, it will be envisaged from the angle of public acceptability for transgenic insect releases. This chapter will consider the reasons for envisaging such releases, and the role of stakeholder engagement in public perception and acceptability of this technology, through an exploration of the ethical implications of such releases and process of informed decision making involved.

24.2   Why Envisage the Potential Release of Transgenic Insects?

There is a growing need to develop new and complementary tools to control disease vectors (Feachem *et al.*, 2019), pests (Savary *et al.*, 2019) and invasive species (IPBES, 2019). Accounting for 17% of the global burden of communicable diseases, vector-borne diseases primarily affect poor communities, claiming more than 700,000 lives every year, mainly in tropical and subtropical areas (WHO, 2017b). Crop losses due to arthropods is estimated to be 18–26% of the annual crop production worldwide and more than US$470 billion, which is a significant economic loss but also a threat to food security for many countries (Culliney, 2014). Similarly, invasive alien species (IAS) are a leading cause of species extinction (Clavero and García-Berthou, 2005). While rodents represent a large part of this threat, insects have been ultimately responsible for the extinction of many bird species, for instance on the islands of Hawaii (Redford *et al.*, 2019), through being vectors of diseases such as avian malaria. There is, therefore, an urgency to address those vectors, pests and IAS effectively and sustainably. Several of the 17 Sustainable Development Goals set up by the United Nations (UN) General Assembly and adopted by all UN Member States in 2015 (available at https://www.un.org/sustainabledevelopment/, accessed 2 April 2022) will not be achieved without the aid of transformative tools to tackle those challenges.

Taking the example of the public health sector, the World Health Organization (WHO) has repeatedly called for research and development of new tools to fight malaria and neglected tropical diseases, stating that 'innovations for new tools, technologies and approaches' are part of the organization's strategy for vector control response (WHO, 2017a).

The WHO strategy for vector control highlights numerous challenges. These include systemic problems in sustaining existing vector control interventions and achieving long-lasting results (many insect vectors have developed resistance to traditional insecticides), compounded by the impacts of climate change in increasing the distribution of disease vectors and insufficient funding levels to provide universal coverage of populations at risk with adequate tools. In addition,

there is a challenge in accessing adequate and timely information to adapt the vector control interventions, for instance to confront growing insecticide resistance (WHO, 2016; Tokponnon *et al.*, 2019).

The development of new tools is needed to overcome challenging problems. The socio-ethical considerations of pesticide use and their sometimes uncertain consequences for people and the environment, or even difficulties in the adoption of benign tools such as bednets, are a compelling justification. Tools like bednets, indoor insecticide residual spraying or larval management have faced important socio-economic and cultural challenges regarding access to the commodities by marginalized communities and behaviour change requirements for effective adoption and use of the tools (Pulford *et al.*, 2011; Kabaghe *et al.*, 2018). Minimal impacts, maximal efficacy of vector control with minimal required socio-cultural investments are characteristics of the innovation that is needed to solve these complex issues. Genetic-based approaches, such as the use of genetically modified insects, including those with engineered gene drive, have been considered a promising avenue to complement interventions for insect vector control (*African Union/NEPAD*, 2018; WHO, 2020a) and a potentially transformative tool in the malaria elimination effort. The acceleration and improvement of the understanding of molecular mechanisms and the discovery of new and effective editing tools such as CRISPR/Cas9 have increased the amount, scope and feasibility of the research on transgenic insects in recent years.

Similar arguments have been made for the need to develop new tools for managing IAS, as they are causing economic losses estimated as an annual mean cost of US$26.8 billion (Diagne *et al.*, 2021) and leading to substantial biodiversity declines and extinctions, particularly on islands. Globally, 86% of extinctions on islands have been attributed to IAS (Bellard *et al.*, 2016). With plenty of global and national policy support focused on the eradication, control, prevention, early detection and rapid response to introductions of IAS, efforts on the ground are not staying abreast of the global threats

(CBD, 2018). The reasons for the lack of investments and failures to stem the establishment of IAS, or reverse the threats posed by IAS, are multifaceted, but most certainly are in part due to the inadequacies of the tools that can be deployed at scale, or because the strategies for their use are not well understood or adopted; in effect the available tools are limiting. For example, the commensal rodents have a global distribution, found on about 85% of islands worldwide, and on all the continents except Antarctica, with massive negative implications for global biodiversity, food security, health and general wellbeing of people. While manual trapping for rodent control efforts can be effective on small sites, the only approach known to be effective in areas any larger than a football field is the use of the rodenticides, primarily the anticoagulant rodenticides. These cause mortality through exsanguination (bleeding out) and are toxic to birds and to other mammals. They are known to enter the food chain, can persist in various environmental compartments for many months to years, and have been demonstrated to lead to poisoning of non-target birds and mammals. While these tools can be effective in controlling rodents, their use at scale is being challenged. The cost of deployment can be quite high, especially with the use of an aerial broadcasting approach, which also limits the scale at which these tools can be used. Their use is increasingly becoming restricted through regulation due to increasing awareness of animal welfare issues from bleeding out, impacts upon non-target species and lack of general social acceptance, particularly in inhabited areas with children, pets and domestic animals that can be at risk of exposure from the use of the anticoagulants.

In the field of agriculture, calls for a reduction in pesticide use are multiplying in light of renewed efforts to promote sustainable agriculture compatible with biodiversity. For instance, the recent European Union Biodiversity Strategy for 2030 calls for a reduction in the use of chemical pesticides by 50% (European Commission, 2020). To achieve this ambitious target, new approaches and tools will be required to ensure that the

pests are controlled and that the conservation of biodiversity can be achieved while preventing crop losses and impacts on food security.

24.3 The Importance of Engagement in the Research Process

When a research project moves from being purely laboratory work to potential field evaluation and implementation of the research outcomes, the project goes through a process of translational science, whereby the initial discovery needs to be adapted or translated for a specific context. In the case of transgenic insects, this involves taking a genetically modified strain of insects from what is usually a containment facility to a field evaluation. In that process, the engagement of affected communities, interested stakeholders and more broadly the 'public' is extremely important (Michener *et al.*, 2012), in particular to understand the factors affecting public acceptability of the proposed technology and to be responsive to public concerns and build a mutual understanding (Cisnetto and Barlow, 2020). These three levels of population (Fig. 24.1) are described by NASEM as follows: (i) communities are 'groups of people who live near enough to a potential field trial or release site that they have a tangible and immediate interest in the project'; (ii) stakeholders are those who have 'professional or personal interests sufficient to justify engagement, but may not have geographical proximity to a potential release site'; and (iii) publics (considered here in plural because these are not a homogeneous group) as representing 'groups who lack the direct connection to a project but nonetheless have interests, concerns, hopes, fears and values that contribute to democratic decision making.'
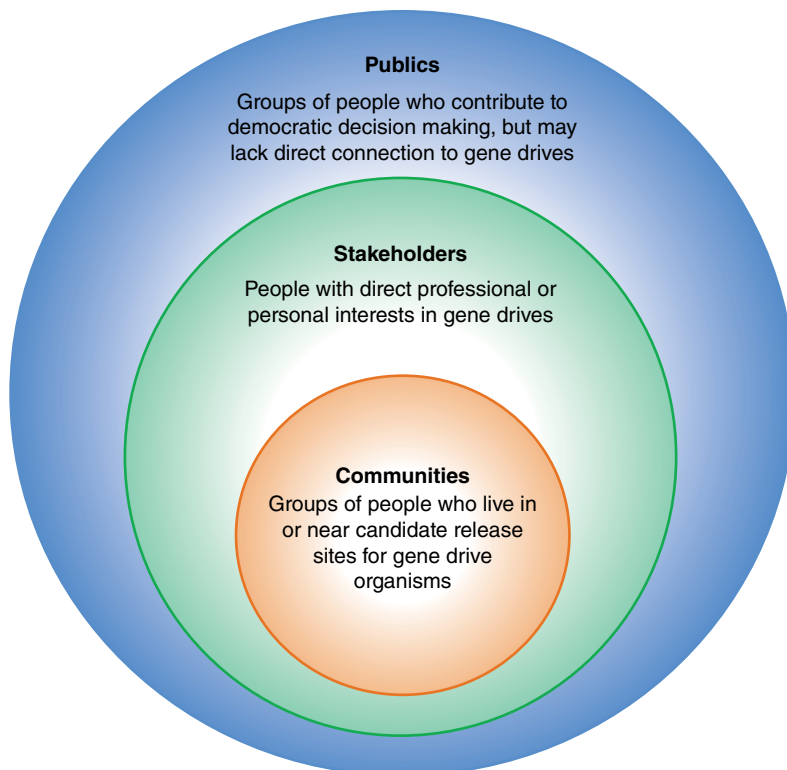
Fig. 24.1. NASEM definitions of communities, stakeholders and publics.

Applying these definitions to large, area-wide applications of transgenic insects requires continuous evaluation and assessment of engagement activities and strategies as the technology moves through phases of development to field evaluation. The engagement required during early scoping or baseline study phases – for instance when a project analyses the wild population of the target species – could be very different from the engagement required before and during a field evaluation. As well as the difference in intensity, one can also expect changes in the population engaged in terms of scope, breadth and duration. The relevant audiences may expand, subgroups may appear and distinctions between groups may become more or less focused over time. It is important to anticipate that there will be a need for flexibility to respond to these dynamic social realities and, as a result, the ability to make regular adjustments, revisions and improvements to engagement strategies over time.

Despite existing experiences with sterile insect technique (SIT), releases for pest control or for vector-borne diseases (Townson, 2009), there is no consensus yet on best practices for engagement for the research on transgenic insects. In the field of public health there has recently been growing attention paid to this topic and a growing body of recommendations specific to the testing of novel technologies for vector control, largely focusing on transgenic insects (WHO, 2012a; WHO/TDR 2014; Ramsey *et al.*, 2014; NASEM, 2016; Thizy *et al.*, 2019; Kormos *et al.*, 2021). This expanding body of work demonstrates increasing awareness among the research community and policymakers about the importance of engaging stakeholders at different levels, from communities to publics. The challenges of achieving universal coverage for well-established interventions such as vaccinations (Wassilak and Orenstein, 2010; Kalantari *et al.*, 2020) as well as the lessons learned from the GM crop experience (Juma, 2016) have led many researchers and funders of research to consider the question of public acceptability of research as a critical part of the research and development process. The assumption that the efficacy of a tool against an identified challenge or the provision of appropriate information is sufficient to guarantee public acceptability (the 'information deficit model') (Sturgis and Allum, 2004) has long been refuted (Bubela *et al.*, 2009).

24.3.1   From an instrumental engagement to a trustful dialogue

There are multiple practical and ethical reasons for involving stakeholders, communities and diverse publics in decisions about the research and future deployment of novel biotechnologies like transgenic insects, and these often intersect (Reed and Curzon, 2015).

While engagement initiatives are mostly well intentioned, historically their execution has been under-resourced and driven by the values and goals of a limited range of individuals and their experiences (Jones, 2014; Reynolds and Sariola, 2018; Stirling *et al.*, 2018). For example, it has not been uncommon for engagement goals to be exclusively driven by a fear of perceived public opposition to innovation rather than a genuine desire to establish a dialogue to feed into the innovation process (Marris, 2015; Carter *et al.*, 2021).

An important question often overlooked in discussions about stakeholder engagement is to decide on whether engagement is intended simply to collect information from respondents for science production or planning, or as a collaborative endeavour for bringing about change; whether it is focused simply on getting consent for a particular study or research programme, or there are instead multiple motivations for that engagement that might even vary and evolve during the research process. Bringing clarity to the purpose of engaging with stakeholders about research that will involve, affect, or is of interest to diverse groups is a critical step in any engagement strategy (Kokotovich *et al.*, 2020).

More recent recommendations consider the importance of co-development (Thizy *et al.*, 2019), co-production (WHO, 2019) and decentralized decision making (Kormos *et al.*, 2021) in the development of engagement

programmes and strategies. Evolving public perceptions and attitudes about science and technology, and the increasing expectation that communities should be involved early in the decision making surrounding the science, continue to drive the growing body of work describing engagement strategies and best practices (Thizy *et al.*, 2019; Costa *et al.*, 2021; Kormos *et al.*, 2021; WHO, 2021b).

More deliberative engagement processes are now encouraged to consider which voices and knowledge types are included in engagement interactions, how the knowledge produced is shared and used, and what values and intentions drive the engagement process itself (NASEM, 2016; Hartley *et al.*, 2019; Kokotovich *et al.*, 2020). Understanding the cultural, historical and institutional influences on how communities perceive, participate in and respond to initiatives proposing the release of novel biotechnologies is also core to an ethical science practice (Thizy *et al.*, 2019).

While employing some level of science translation to engage others about science can be a necessary component of successfully engaging with communities, it is no longer sufficient to translate complex science concepts for gaining trust, confidence or approval for interventions (Carmichael *et al.*, 2015; Wanyama Chemonges *et al.*, 2021). Co-creation models of engagement where multiple knowledge types, values and aspirations are taken into consideration are guiding responsible science practice (Hartley *et al.*, 2019; Barnhill-Dilling and Delborne, 2021).

24.3.2 Public perception and decision making

The public (as defined by NASEM, 2016) as a stakeholder in the conversations around public acceptability of genetic technologies may be a group that has limited technical knowledge about a particular innovation. However, they have a great deal of tacit knowledge about how innovation might translate on the ground and how it might affect individuals and communities. Therefore, citizens play an important role in contextualizing novel genetic innovations.

When discussing public attitudes, perceptions and acceptability about novel genetic technologies, the core question is how people interpret the technology through their personal lens and how people subsequently define their views about it. Past social research on genetic technologies has highlighted that many people are risk-averse and wary of negative consequences, even in the absence of risk data (Kraemer, 2010). It is a natural human reaction to be wary or fearful of something new, particularly something that is perceived as challenging our very sense of normality or naturalness (Carter *et al.*, 2021). Often, when people make these assessments of risks and benefits about genetic technologies, they do so in a way that may not be based on fact or rationality or even coherence.

Media coverage plays a role in shaping those perceptions, firstly by creating an awareness and as such a potential societal dialogue about the technology proposed. Without media coverage, the public at large is often unaware of technological developments and therefore is not part of their development (Bauer *et al.*, 2001). This public awareness derived via media may be attended by issues such as hype around a technology that is still in its infancy before an evidence-based dialogue can occur (Caulfield, 2005). Secondly, media coverage can play a role in providing information that could influence the public's perception about a technology, its potential risks and its benefits (Teisl *et al.*, 2009). However, it would be oversimplistic to think that better media coverage and providing information would be sufficient to shift the public's perception about a technology. In fact, the psychological literature consistently demonstrates the subjective nature of how humans perceive and assess risks and the role that underlying personal values play in those assessments. One of the main reasons humans rely on emotions to assess risk is that emotions can serve as decision-making 'shortcuts', which can be especially useful under conditions of insufficient knowledge and if additional information to help in decision making is unavailable. Emotions are not only feelings; they also carry with them an

inherent informational component that guides the formation of an attitudinal judgement towards an object or event, notably if other appraisal pathways are lacking, for example technical knowledge (Schwarz and Clore, 1983; Slovic, 1999; Slovic *et al*., 2004). Importantly, this fact does not make public perceptions of risks and benefits any less important or influential. Instead, this highlights the complexity of human decision making, particularly in the context of novel genetic technologies – most of which elicit an almost reflexive emotional response from people. Interestingly, more recent research on public attitudes about synthetic biology has shown that the role of negative emotions (e.g., concern, anger, fear), while influential, did not match the stronger influence of positive emotions (e.g., hope, excitement, curiosity) in predicting support for novel genetic technologies (Mankad *et al*., 2020). In practice, this may have implications for how a technology is framed to its intended audience, how communication about the science is articulated, and how trustworthy the source of that information is perceived to be.

Another important consideration for the inclusion of diverse public perspectives is that different people articulate a single problem differently. Therefore, the way a scientist or institutional stakeholder may perceive the problem (and its associated genetic solution) is very different from how a community member may define the same problem (and desired solution). This undoubtedly influences how personal decisions are made. Sometimes, the public may simply be apathetic to a problem that may have significant and pervasive environmental and sustainability implications. For instance, invasive alien species might not be perceived as a threat to biodiversity but as sources of livelihood, leading to conflict about their management and elimination (Kelsch *et al*., 2020). In that case, it is often tempting to introduce more knowledge to a particular problem frame to explain the significance of a solution. However, as the literature has consistently demonstrated, the provision of additional information to redress a knowledge deficit rarely has the intended effect of making people care more; it may simply be that a particular community or group has no interest in engaging about a particular technological innovation, or that embedded cultural values override the decision-making process (Allum *et al*., 2008). Thus, it becomes even more critical to understand the overarching context within which a problem might occur and whether there is demand for a solution.

Therefore, human decision making is very dependent upon the emotional, psychological and contextual influences that guide perception (Finucane *et al*., 2000). Often, people will be more engaged in topics that directly affect them, their family or their community, including technologies that solve health- and conservation-related problems (Hobman *et al*., 2021). There is also a range of other attributes related to innovations considered important in influencing acceptance and decision making around novel genetic innovations. This includes the degree of complexity an innovation may be perceived as having, the compatibility or fit with current solutions being implemented for the same problem, risks related to implementation (and not the technology alone) and access to support resources if the implementation of the technology goes awry (Rogers, 2004). Research in the synthetic biology space suggests that trust in scientists developing the technology and trust in broader regulatory systems also play a role in how the public may perceive genetic interventions (Hobman *et al*., 2021).

24.4 What Ethical Considerations Affect Public Acceptability of Transgenic Insect Releases?

Laboratory research to develop gene drive mosquitoes, their potential eventual release into open environments and the releases of genetically modified insects in various parts of the world to fight vector-borne diseases and other challenges are accompanied by lively ethical debates that affect public perception and acceptability of transgenic insect releases. Engaging with these ethical debates is critical

for researchers to inform their practice and approaches to technology development as well as public dialogue. The capacity of researchers to respond to these ethical questions in their research can be pivotal to public acceptability of transgenic insects.

24.4.1   Novel tech, novel ethics?

It is not uncommon that novel technologies, particularly powerful technologies with the potential for broad impact and consequence, may be viewed as exceptional in several respects. This can impact public acceptability (Juma, 2016). Sometimes attempts are made to ground this exceptionalism in claims that a given technology introduces novel ethical issues or challenges, as they have in gene drive mosquitoes.

However, novel technology is rarely attended by truly novel ethical issues (Newson, 2015). One of the primary reasons that novel technologies are associated with novel ethical issues may be that the different categories of analysis become conflated or are misunderstood. When examining and discussing the merits of new technology, at least three categories of analysis must be distinguished: scientific or technical; philosophical; and political (Altimore, 1982). The questions and issues arising from the scientific or technical category will be quantitative and empirical and will need to be posed to, and answered by, those with relevant scientific expertise. Take as an example the case of engineered gene drive mosquitoes, which present a significant scientific and technical novelty. As such, they demand a large amount of continued research to mitigate the uncertainty surrounding conceptions of their potential environmental release. The philosophical and political questions require consideration of non-quantitative variables such as justice and fairness and other values that inform ethics. However, while engineered gene drive certainly raises several complex challenges in philosophical and political categories, both of which may consider issues commensurate with ethical value, these are not completely new kinds of challenges.

We have faced their kind many times before in other circumstances and have principles and frameworks developed from previously encountered, analogous cases to draw upon and apply in the case of transgenic insects.

24.4.2   The role of humans in nature and the concept of naturalness

Humans across cultures share a strong intuition that the natural world is valuable. Environmental ethicists strive to determine from whence this value derives and how it can be preserved (Palmer, 2002). The non-human world can be valued for instrumental reasons because it provides timber, game, or recreational spaces. It can also be valued intrinsically, for its own sake. Ethicists have put forth different arguments for this value (Palmer, 2002). Some have focused on the diversity of the non-human world, others on its beauty or spiritual significance. However, many have argued that the natural world is valuable precisely because it is 'natural.'

'Naturalness' is often used to talk about the ethical value that a place has in virtue of being free from human control or influence. Because environmental systems and non-human species are thought to be morally valuable because they are free from human control and influence, human interventions into the environment have traditionally been viewed as something that causes a loss of moral value. By intervening in nature, some argue, humans are breaking a causal process that is independent (or at least largely independent) from humans (Rolston, 1989; Katz, 2000; Preston, 2008). In addition, this interference is often criticized as a sign of human hubris, questioning the role of humans in nature. This notion of human hubris comes from Greek mythology and was considered a crime in Ancient Greece, while the Christian tradition has long considered hubris to be a sin. Human hubris is related to a conception of nature and wilderness as a perfect system – what theistic religions would consider God's creation – and therefore the intervention of humans upon nature disrupts what God intended

and amounts to an overstep on humanity's part with regard to their appropriate role in or relationship to nature (Cronon, 1996). This leads to the regular comments about 'playing God' that are often associated with genetic modifications (Dabrock, 2009; Waytz and Young, 2019; Carter *et al*., 2021).

Developing transgenic insects and releasing them into environments to help combat diseases like malaria, Zika or dengue could potentially compromise the presumed naturalness of a particular place or species. For example, transgenic mosquitoes developed in laboratories and then released into the wild would mate with wild populations. As a result, naturalness would be compromised, since the system would have an organism that humans have altered persist in it. Furthermore, if the modification spreads throughout the species, then the naturalness of the species would also be compromised.

Scholars who subscribe to naturalness in their theories tend to be highly sceptical of transgenic manipulations in general, because the technique or method of technology creation itself is sometimes seen as 'unnatural' in a way that traditional breeding or land management is not. However, it seems likely that deploying transgenic insects will not compromise naturalness as much as traditional means of vector control (Callies and Rohwer, 2021). Traditional vector control methods usually involve the draining of wetlands and the widespread use of insecticides, both of which affect a multitude of species beyond the target insect.

If a place has value in terms of being free from human influence, then an ecosystem that has been completely changed (for example, a wetland that has been drained) will lose more of this value than a system where merely one component of that system has been changed by humans (for example, the introduction of transgenic *Anopheles gambiae*).

Even in cases where the goal of the genetic intervention is the localized elimination of the species, there is less value lost in the transgenic case since only one narrowly targeted component would be lost, while the draining of the wetland would result in the loss of every species in that system that is dependent on standing water. This elimination might also be temporary, as the intervention might have a transient impact. This is, for instance, the case of SIT or self-limiting transgenic insects, but even gene drive insects (see Bottino-Rojas and James, Chapter 11; Scott *et al*., Chapter 17, this volume) might have different persistence profiles depending on their molecular characteristics and the interactions with the wild population (North *et al*., 2019). There have been cases of local vector suppression through the traditional vector control interventions, often accompanied with landscape changes (Dash *et al*., 2000). In those circumstances, the influence of humans extends to a broad class of invertebrates, since insecticides kill indiscriminately. In sum, compared with traditional pest controls, transgenic approaches seem to be better at limiting the potentially value-reducing impact that humans may have on natural environments.

Even those who subscribe to strong conceptions of naturalness recognize that reduction in the richness and diversity of nature is justifiable insofar as it necessary to satisfy vital human needs. As such, the pursuit of species elimination may be acceptable only in very narrow circumstances, and with minimal impact on the environment (Rolston, 1985). The conditions that are often proposed by ethicists when considering transgenic insects – and in particular gene drive insects – such as having exhausted all other options, having deliberated the underpinning values of the intervention (EGE, 2021), reflect this tension and the specific contexts in which such applications might be acceptable. Even when environments are valued for being free from human control, this does not necessarily imply that satisfaction of other ethical obligations cannot override this value or supersede it (Callicott, 1992; Mazerik and Rejeski, 2014).

Not every environmental ethicist believes naturalness is valuable (Vogel, 2015; Rohwer and Marris, 2021). Valuing naturalness rests upon a problematic view that humans are not part of nature. Complete and comprehensive environmental ethical frameworks are possible without any reference to naturalness. Environmental ethics that focus on the

value of biodiversity or wildness, or on aesthetic and cultural values, exist and reject the idea that naturalness is valuable. These ethics will or should find the genetic alteration of insects much less problematic, since these sorts of approaches will not compromise these values like traditional vector control strategies.

Finally, the question of whether anything on Earth can still be considered purely natural or wild, considering the extensive interactions between humans and their environment, should be posed and seriously considered. The existence of the 'rewilding' concept and the variety of definitions it offers (Jørgensen, 2015) interrogates the notion of wilderness, and whether it can be recreated by humans. Some conservationists invite us to consider ecosystems in their historical variability in time and space, to understand their evolution, and 'recognize that nature is a flux' influenced by interactions with humans and changes in climate (Gillson and Willis, 2004).

24.4.3 Social justice considerations

How do questions of social justice affect public acceptability of the release of transgenic insects? The question of how to introduce a process for all levels of public (communities, stakeholders and publics) to have a fair chance to participate meaningfully during the decision-making process is central to social justice. There are several power inequities at play in these circumstances.

Inequity can stem from differences in knowledge levels and types, for instance hindering the ability of some to participate in risk assessment processes. These inequities between those with formal scientific knowledge regarding the technology or the ecology and those with either a different type of knowledge (such as traditional knowledge) or a limited knowledge level regarding these matters need to be considered. Differences in social capital and influence within a society and its decision-making processes are another source of inequity that can marginalize those more directly affected by an intervention while benefiting others who are more vocal due to their socio-economic position or political influence. Considering these existing power inequities, a 'level playing-field' for input, such as public comment periods, which are usually limited to a website soliciting public comment, may not truly allow all voices to be heard. This raises questions about procedural justice.

Procedural justice refers to the fairness of the process by which a decision is made (Thibaut and Walker, 1975). This concept is particularly helpful in situations where there might be disagreements between different groups regarding a decision to be made; in such cases, guaranteeing that the process by which the decision is fair is important for the legitimacy of that decision (Syme and Nancarrow, 1991). Consequently, to obtain a fair public consultation process, it is crucial to ensure the process is designed with awareness and consideration of existing societal power dynamics and to mitigate appropriately against the ways in which these dynamics may cause inequity in the process.

Secondly, as with other technological developments, the questions of who is developing the technology and technology transfer, ownership and related capacity development are critical (CBD, 2021a,b). While transgenic insects were originally developed in laboratories in high-income countries, efforts to ensure that these technologies can be understood and owned by researchers and regulators from low- and middle-income countries is critical. These efforts need to be multi-layered, including not only training for researchers and regulators, but also infrastructure development to ensure safe containment of early research and support for policymakers in implementing and strengthening their biosafety framework. It is a question of sovereignty but also an important aspect for public acceptability (Kamwi, 2016).

Thirdly, inequality in access to research and technology is a concern surrounding development and application of transgenic insect technologies. Most discoveries and laboratory development occur in high-income countries, whereas field testing and deployment are envisioned primarily in low-income

countries. This is partially because the challenges they intend to address (such as vector-borne diseases) are mostly prevalent in those low-income countries. Local power inequities (e.g., class, gender, ethnicity, race) also surface to complicate engagement and decision making about novel technology research and evaluation. The failure to satisfy elements of procedural justice in decision making is likely to affect public acceptability of the technology.

Social justice entails much more than just 'good' and 'net benefits' to society. For example, public health and conservation applications of transgenic insects could deliver collective benefits with minimal risks (or costs), but those outcomes may be distributed inequitably. Attending to those distributions – and even how different segments of societies identify consequences as negative or positive (Barnhill-Dilling and Delborne, 2021) – will require careful consideration of minority viewpoints and perspectives.

Because technologies are rarely neutral (Winner, 1980), genetic approaches could alter social relations in ways that extend beyond 'fixing the problem' of, for example, malaria or biodiversity loss or crop damage. Transgenic insects could empower some actors, displace some technologies, and transform how humans experience their environment.

Attending to considerations of justice in these broader terms will require robust engagement with stakeholders and publics (WHO, 2021b), and acceptance of the technology will depend on satisfying these various dimensions of justice, as well as the satisfaction of other ethical obligations.

24.5 Achieving an Informed Decision About the Release of Transgenic Insects

24.5.1 Whose decision?

Despite calls for 'science-based' regulations, decisions to allow the release of transgenic insects will be unavoidably political. In the current situation, for most countries the first step to this decision making is a risk assessment done by the national biosafety authority. This leads to an assessment of the potential risks and of their management as proposed in the research protocol under review. This assessment usually goes to another decision-making body that weighs the risk assessment against other considerations such as potential benefits, values, national priorities and public acceptability. Scientific evidence regarding risks and potential benefits is part of the decision but is not the only factor in this decision (Sarewitz, 2004; Pielke, 2007). Knowing this, the focus of the governance decision can be on how to make good decisions (which will necessarily be informed by a mix of facts and values) about releasing transgenic insects.

Others have explored the gaps, uncertainties and opportunities for the formal governance of emerging biotechnologies such as transgenic insects (Hartley *et al*., 2016; NASEM, 2016; Evans and Palmer, 2018; Redford *et al*., 2019; Thizy *et al*., 2020; Reynolds, 2021) (see Beech *et al*., Chapter 25; Pereira, Chapter 27; Hayes and Quinlan, Chapter 28, this volume). In addition to these important questions, attention should be drawn to how communities, stakeholders and publics can be engaged throughout the development and evaluation of transgenic insects research (Lavery *et al*., 2010; Burgess *et al*., 2018; Stirling *et al*., 2018; Thizy *et al*., 2019). Such work enables mutual learning among people with different sources and kinds of expertise, two-way communication that creates understanding about interests and values, and trust-building. Communities, stakeholders and publics have a role to play in the good governance and design of responsible research frameworks. However, researchers and decision-making bodies (e.g., regulatory authorities, government) have different obligations towards each of these groups.

The recently published second edition of the 'Guidance Framework for Testing Genetically Modified Mosquitoes' by the World Health Organization (WHO, 2021a) offers some clarity on the different levels of engagement and authorization appropriately sought from each of these groups.

It distinguishes the obligations to seek consent or community authorization for individuals or communities directly who may be affected by the research, and the need for consideration for other stakeholders' or publics' concerns and interests in a respectful manner. Those differences are critical, as they shape the framework for different populations' participation in the research process. Failing to identify or to recognize those levels can lead to an erroneous perception that all populations or stakeholders ought to have an equal say in whether the research should proceed or not. Some researchers argue that there is an ethical obligation to focus the engagement process in the communities most directly affected by those potential releases as well as the burden they are trying to combat, regardless of their political or social capital (Roberts and Thizy, 2021). There are critical questions of social justice which bear on decisions made about whom to engage and ask for consent or authorization, and how this engagement is carried out. Operational issues, such as the tools, language, venues and timings used, influence the ability of certain groups to participate in these engagement and decision-making processes. As such, procedures that ensure just and fair access to the communities most affected by these releases are critical.

24.5.2 Informed consent and community authorization for the experimental release of transgenic insects

Public health is probably the sector where the discussion about the type of 'consent' (here used in the broad definition of the term, as the fact for an individual or a group to give permission for something to happen) that is required prior to the experimental release of transgenic insects is the most advanced and formalized. There is an important distinction to make between the research phase (experimental releases) and the potential use of the technology (potential roll-out of gene drive through large-scale releases). Ethical research guidelines safeguard individual

integrity and rights during the research phase, while an intervention is being evaluated for its safety and effectiveness. Within its mandate to determine standards and guidance to ensure responsible research on public health issues, the WHO has established a clear framework for consent. This framework distinguishes the 'human research subject' from other participants. Research involving human subjects requires individual informed consent (CIOMS, 2016). For the WHO, 'the ethical foundation of informed consent is the principle of respect for persons. Competent individuals are entitled to choose freely whether to participate in research and to make decisions based on an adequate understanding of what the research entails' (WHO, 2012). The guidelines of the Council for International Organizations of Medical Sciences (CIOMS) provide particular considerations and standards for informed consent, including the type of information that should be disclosed to the individual to allow them to make an informed decision (CIOMS, 2016).

The release of transgenic insects can be qualified as an 'area-wide intervention', meaning that the impacts (whether positive or negative) will be experienced by the whole population living in the area of potential impact without differentiation of their participation status to the study. In other words, individuals cannot opt out from participating in the research unless they leave the area where this research is taking place. This is the fundamental ethical and practical challenge of transgenic insects, even though it is not entirely a new ethical challenge, as other public health interventions (for example water fluoridation, aerial insecticide spraying) have faced similar questions.

Due to the complexities of transgenic insects as area-wide interventions, significant efforts have been spent by policymakers and researchers to think about the appropriate consent framework for those releases. A broad consensus emerged to assert that living in the area of release of such organisms does not automatically mean that those residents are human subjects (NASEM, 2016; Kolopack and Lavery, 2017; Singh, 2019; WHO, 2020b). Individual informed consent would only be required when data

or samples would be collected from an individual, for instance if the study is looking at epidemiological results of the intervention and requires blood samples from a particular population (WHO/TDR, 2014; James *et al.*, 2018; WHO, 2020b). Therefore, various guidelines and literature refer to community agreement or community authorization as the viable analogue to informed consent for the release of transgenic insects during the research phase (WHO/TDR, 2014; NASEM, 2016; Kolopack and Lavery, 2017; Singh, 2019; WHO, 2021a).

There remain many unanswered questions. What legitimately constitutes community authorization? How should it be sought (from whom, at what point along the research pathway, on the basis of what information), recorded, implemented and monitored? While the literature and guidance about the ethical requirements for such authorization are numerous, as previously referenced, descriptions of detailed procedures for how this ought to be done in practice remain to be seen. Decades of work and learnings on individual informed consent are documented and available for researchers, yet it is still difficult to 'get it right'. For researchers committed to setting a just and legitimate process for community authorization, examples of documented good practices are rare (Kolopack *et al.*, 2015; Costa *et al.*, 2021). The international frameworks on releases of living modified organisms, such as transgenic insects, tend to be silent about researchers' and other actors' different obligations regarding community engagement, participation and authorization process, focusing mainly on regulatory authorities' obligations (CBD, 2000).

The primary oversight for this aspect of research comes from Research Ethics Committees (RECs) (whether institutional or national) and/or Institutional Review Boards (IRBs), who are more experienced with the individual informed consent framework than community authorization. These committees are responsible for ensuring that the process proposed addresses researchers' ethical obligations to respect communities' interests, respond to their collective concerns, and provide a fair and just process for making decisions. For typical research, the researchers' obligations are to draft the protocol, submit it to an REC, adapt it according to potential REC recommendations, and then implement it as such. In most cases, this is sufficient to provide researchers with legitimacy, as the REC's approval validates that the process is ethical, and this approval is usually subject to further verifications from the committee upon implementation. However, in the case of transgenic insects, this may no longer be sufficient. In the absence of established and recognized standards in this field, research projects are vulnerable to criticisms regarding the legitimacy of their processes. They can have difficulty evaluating if those analyses are rooted in a fundamental disagreement about the nature of the technology or about an issue of procedural justice related to the community agreement/authorization model.

Scientific researchers cannot be left alone to examine these complex issues related to consent and community agreement. A framework and standards are required to help researchers navigate the legitimacy of their process. Box 24.1 highlights some of these key considerations for the legitimacy of decision-making processes. Considering the diversity of contexts and applications, national or regional standards and frameworks for each sector (i.e., public health, agriculture and conservation) would probably be more appropriate than trying to establish a global standard, which would no doubt fail to capture the necessary contextual diversity and local socio-cultural specificities. In recent years, in the absence of such standards, social scientists and engagement practitioners involved with such research have identified and codified good practices and created a community of practice on this topic (Kolopack *et al.*, 2015; Bartumeus *et al.*, 2019; Costa *et al.*, 2021; Kormos *et al.*, 2021; Pare Toe *et al.*, 2021; Thizy *et al.*, 2021).

24.5.3   Where does community agreement fit in the decision-making process?

In discussing community agreement, it is important to remember that this process is

---

Box 24.1. Key legitimacy questions to consider for community agreement for transgenic insect releases.

- How is the community defined, and by whom?
- Which community members participate in decision making and why? How do you achieve representativeness?
- What information is provided to the community prior to their decision? Who decides it is appropriate and sufficient?
- Who provides that information and in what form?
- How does the engagement model address the power imbalances to ensure a fair and equitable decision-making process?
- Should community understanding be assessed prior to their decision and if so by whom?
- How to balance communities' rights to self-determine what their governance system is and the aspiration for more inclusivity of marginalized groups?
- Are there any external mechanisms to control the community agreement?
- How does the community agreement get evaluated? And by whom?

---

only part of the national decision-making process. Communities and publics are consulted about decision making. Researchers must seek and obtain community agreement before releasing transgenic insects for research purposes, as part of requirements for ethical research process. Public consultation as part of the regulatory process before decision making by the national authorities is often an obligation stipulated in the national legislations or frameworks. This requirement is usually a transcription of the Cartagena Protocol article 23 on public participation, for countries signatory to the Protocol (see Pereira, Chapter 27, this volume). This article states, 'The Parties shall, in accordance with their respective laws and regulations, consult the public in the decision-making process regarding living modified organisms and shall make the results of such decisions available to the public' (CBD, 2000). While the text calls for public consultation, it also specifies that those will be done according to national laws and regulations, leaving lots of flexibility to the Parties (countries) as to the manner in which they choose to carry out such consultations and the weight they give them in their decision making.

However, the Cartagena Protocol (and its transcription into national regulations for Parties that are signatories to the Protocol) explicitly puts the responsibility of public consultation on the national authorities as part of their decision-making process for living modified organisms. The two levels of consultation – from research projects as part of their ethical obligations and national authorities as part of their regulatory framework – are complementary but profoundly different. The consultation from national authorities is part of their decision-making process on whether or not to grant a permit for the proposed research protocol or the use of the proposed technology. At the same time, for the research phase where this is applicable, the agreement obtained by the researchers from the community may not be legally binding or related to any regulatory process of approval, despite its ethical importance. However, various analyses demonstrate how those public consultation processes led by governments might be inadequate (Ahteensuu and Siipi, 2009; Hartley and Millar, 2014; Ledingham and Hartley, 2021). These consultations are restricted to a case-by-case scientific risk assessment process and are not designed to examine broader sets of socio-economic, cultural issues, or the question of potential benefits from the envisaged release.

References

African Union/NEPAD (2018) *Gene Drives for Malaria Control and Elimination in Africa*. Report of the High-Level African Union Panel on Emerging Technologies (APET). *African Union Development Agency (AUDA) and New Partnership for Africa's Development (NEPAD)*, Midrand, Gauteng, South Africa.

Ahteensuu, M. and Siipi, H. (2009) A critical assessment of public consultations on GMOs in the European Union. *Environmental Values* 18, 129–152. doi: 10.3197/096327109X438035

Allum, N., Sturgis, P., Tabourazi, D. and Brunton-Smith, I. (2008) Science knowledge and attitudes across cultures: a meta-analysis. *Public Understanding of Science* 17(1), 35–54. doi: 10.1177/0963662506070159

Altimore, M. (1982) The social construction of a scientific controversy: comments on press coverage of the recombinant DNA debate. *Science, Technology, & Human Values* 7(4), 24–31. doi: 10.1177/016224398200700404

Barnhill-Dilling, S.K. and Delborne, J.A. (2021) Whose intentions? What consequences? Interrogating 'Intended Consequences' for conservation with environmental biotechnology. *Conservation Science and Practice* 3(4), e406. doi: 10.1111/csp2.406

Bartumeus, F., Costa, G.B., Eritja, R., Kelly, A.H., Finda, M. *et al.* (2019) Sustainable innovation in vector control requires strong partnerships with communities. *PLOS Neglected Tropical Diseases* 13(4), e0007204. doi: 10.1371/journal.pntd.0007204

Bauer, M., Kohring, M., Allansdottir, A. and Gutteling, J. (2001) The dramatisation of biotechnology in elite mass media. In: Gaskell, G. and Bauer, M.W. (eds) *Biotechnology 1996–2000: The Years of Controversy*. Science Museum, London, pp. 35–52.

Bellard, C., Cassey, P. and Blackburn, T.M. (2016) Alien species as a driver of recent extinctions. *Biology Letters* 12(2), 20150623.

Bubela, T., Nisbet, M.C., Borchelt, R., Brunger, F., Critchley, C. *et al.* (2009) Science communication reconsidered. *Nature Biotechnology* 27(6), 514–518. doi: 10.1038/nbt0609-514

Buchthal, J., Evans, S.W., Lunshof, J., Telford, S.R. and Esvelt, K.M. (2019) Mice Against Ticks: an experimental community-guided effort to prevent tick-borne disease by altering the shared environment. *Philosophical Transactions of the Royal Society B: Biological Sciences* 374(1772), 20180105. doi: 10.1098/rstb.2018.0105

Burgess, M.M., Mumford, J.D. and Lavery, J.V. (2018) Public engagement pathways for emerging GM insect technologies. *BMC Proceedings* 12(8), 12. doi: 10.1186/s12919-018-0109-x

Callicott, J.B. (1992) Rolston on Intrinsic Value. *Environmental Ethics* 14(2), 129–143. doi: 10.5840/enviroethics199214229

Callies, D.E. and Rohwer, Y. (2021) Justifying an intentional species extinction: the case of *Anopheles gambiae*. *Environmental Values* 31, 193–210. doi: 10.3197/096327121x16081160834786

Carmichael, R.H., Botton, M.L., Shin, P.K.S. and Cheung, S.G. (eds) (2015) *Changing Global Perspectives on Horseshoe Crab Biology, Conservation and Management*. Springer Nature, Cham, Switzerland. doi: 10.1007/978-3-319-19542-1

Carter, L., Mankad, A., Hobman, E.V. and Porter, N.B. (2021) Playing God and tampering with nature: popular labels for real concerns in synthetic biology. *Transgenic Research* 30(2), 155–167. doi: 10.1007/s11248-021-00233-2

Caulfield, T. (2005) Popular media, biotechnology, and the 'Cycle of Hype'. *Houston Journal of Health Law & Policy* 337(2004), 213–233.

CBD (2000) *Cartagena Protocol on Biosafety – Text and Annexes. 1–30.* Convention on Biological Diversity, Montreal. Available at: https://bch.cbd.int/protocol/text/ (accessed April 2022)

CBD (2018) Decision adopted by the Conference of the Parties to the Convention on Biological Diversity 14/11. Invasive alien species, 30 November 2018, CBD/COP/DEC/14/11. 2018(November), 1–8. Convention on Biological Diversity, Montreal. Available at: https://www.cbd.int/doc/decisions/cop-14/cop-14-dec-11-en.pdf (accessed April 2022)

CBD (2021a) *Draft report of the Subsidiary Body on Scientific, Technical and Technological Advice on its twenty-fourth meeting (part 1)*. *Convention on Biological Diversity, Montreal. Available at*: https://www.cbd.int/doc/sbstta-24-part1-I-01-en.pdf (accessed April 2022)

CBD (2021b) Report of the Subsidiary Body on Implementation on its third meeting: account of proceedings of the first part of the meeting (part 1) (Issue June). Available at: https://www.cbd.int/doc/c/61f4/eebe/0b12f09771373d2edbb5ae14/sbi-03-part1-l-01-rev1-en.pdf (accessed April 2022)

Cisnetto, V. and Barlow, J. (2020) The development of complex and controversial innovations. Genetically modified mosquitoes for malaria eradication. *Research Policy* 49(3), 103917. doi: 10.1016/j.respol.2019.103917

Clavero, M. and García-Berthou, E. (2005) Invasive species are a leading cause of animal extinctions. *Trends in Ecology & Evolution* 20(3), 110.

Costa, G.B., Smithyman, R., O'Neill, S.L. and Moreira, L.A. (2021) How to engage communities on a large scale? Lessons from World Mosquito Program in Rio de Janeiro, Brazil [version 2; peer review: 2 approved, 1 approved with reservations]. *Gates Open Research* 4(109). doi: 10.12688/gatesopenres.13153.2

CIOMS (2016) *International Ethical Guidelines for Health-related Research Involving Humans*, 4th edn. Council for International Organizations of Medical Sciences, Geneva. Available at: https://www.cioms.ch/wp-content/uploads/2017/01/WEB-CIOMS-EthicalGuidelines.pdf (accessed April 2022)

Cronon, W. (1996) The trouble with wilderness: or, getting back to the wrong nature. *Environmental History* 1(1), 7–28. doi: 10.2307/3985059

Culliney, T.W. (2014) Crop losses to arthropods. In: Pimentel, D. and Peshin, R. (eds) *Integrated Pest Management: Pesticide Problems*, Vol. 3. Springer, Dordrecht, Netherlands, pp. 201–225. doi: 10.1007/978-94-007-7796-5_8

Dabrock, P. (2009). Playing God? Synthetic biology as a theological and ethical challenge. *Systems and Synthetic Biology* 3(1), 47–54. doi: 10.1007/s11693-009-9028-5

Dash, A.P., Hazra, R.K., Mahapatra, N. and Tripathy, H.K. (2000) Disappearance of malaria vector *Anopheles sundaicus* from Chilika Lake area of Orissa State in India. *Medical and Veterinary Entomology* 14(4), 445–449. doi: https://doi.org/10.1046/j.1365-2915.2000.00265.x

Diagne, C., Leroy, B., Vaissière, A.-C., Gozlan, R.E., Roiz, D. *et al.* (2021) High and rising economic costs of biological invasions worldwide. *Nature* 592(7855), 571–576. doi: 10.1038/s41586-021-03405-6

EFSA (European Food Safety Authority), Devos, Y., Gallani, B. and Firbank, L.G. (2020) Stakeholder workshop 'Problem formulation for the environmental risk assessment of gene drive modified insects' (15 May 2019, Brussels). *EFSA Supporting Publications* 17(3), 1819E. doi: 10.2903/sp.efsa.2020. EN-1819

EGE (2021) *Ethics of Genome Editing*. Opinion no. 32 of the European Group on Ethics in Science and New Technologies to the European Commission, Brussels. doi: 10.2777/763

European Commission (2020) *Bringing Nature Back into Our Lives. EU Biodiversity Strategy for 2030*. Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions. Brussels, Belgium.

Evans, S.W. and Palmer, M.J. (2018) Anomaly handling and the politics of gene drives. *Journal of Responsible Innovation*, 5 (Sup.1), S223–S242. doi: 10.1080/23299460.2017.1407911

Feachem, R.G.A.A., Chen, I., Akbari, O., Bertozzi-Villa, A., Bhatt, S. *et al.* (2019) Malaria eradication within a generation: ambitious, achievable, and necessary. *The Lancet* 394(10203), 1056–1112. doi: 10.1016/S0140-6736(19)31139-0

Finucane, M.L., Alhakami, A., Slovic, P. and Johnson, S.M. (2000) The affect heuristic in judgments of risks and benefits. *Journal of Behavioral Decision Making* 13(1), 1–17. doi: 10.1002/(SICI)1099-0771(200001/03)13:1<1::AID-BDM333>3.0.CO;2-S

Gillson, L. and Willis, K.J. (2004) 'As Earth's testimonies tell': wilderness conservation in a changing world. *Ecology Letters* 7(10), 990–998. doi: 10.1111/j.1461-0248.2004.00658.x

Hartley, S. and Millar, K. (2014) The challenges of consulting the public on science policy: examining the development of European risk assessment policy for genetically modified animals. *Review of Policy Research* 31. doi: 10.1111/ropr.12102

Hartley, S., Gillund, F., van Hove, L. and Wickson, F. (2016) Essential features of responsible governance of agricultural biotechnology. *PLoS Biology* 14(5), e1002453. doi: 10.1371/journal.pbio.1002453

Hartley, S., Thizy, D., Ledingham, K., Coulibaly, M., Diabaté, A. *et al.* (2019) Knowledge engagement in gene drive research for malaria control. *PLoS Neglected Tropical Diseases* 13(4), e0007233. doi: 10.1371/journal.pntd.0007233

Hobman, E.V., Mankad, A. and Carter, L. (2021) Public attitudes towards synthetic biology. CSIRO Synthetic Biology Future Science Platform, *Canberra. Available at*: https://research.csiro.au/synthetic-biology-fsp/public-attitudes/ (accessed April 2022)

IPBES (2019) Summary for policymakers of the global assessment report on biodiversity and ecosystem services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services. *Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services, Bonn, Germany.* doi: 10.5281/zenodo.3553579.

James, S., Collins, F.H., Welkhoff, P.A., Emerson, C., Godfray, H.C.J. *et al.* (2018) Pathway to deployment of gene drive mosquitoes as a potential biocontrol tool for elimination of malaria in Sub-Saharan Africa: Recommendations of a Scientific Working Group. *American Journal of Tropical Medicine and Hygiene*, 98 (Suppl. 6), 1–49. doi: 10.4269/ajtmh.18-0083

Jones, R.A.L. (2014) Reflecting on public engagement and science policy. *Public Understanding of Science* 23(1). doi: 10.1177/0963662513482614

Jørgensen, D. (2015). Rethinking rewilding. *Geoforum*, 65, 482–488. doi: 10.1016/j.geoforum.2014.11.016

Juma, C. (2016) *Innovation and its Enemies: Why People Resist New Technologies*. Oxford University Press, Oxford, UK. doi: 10.1093/acprof:oso/9780190467036.001.0001

Kabaghe, A.N., Chipeta, M.G., McCann, R.S., Terlouw, D.J., Tizifa, T. *et al.* (2018) Access and adequate utilization of malaria control interventions in rural Malawi: a descriptive quantitative study. *Malaria Journal* 17(1), 104. doi: 10.1186/s12936-018-2253-1

Kalantari, N., Borisch, B. and Lomazzi, M. (2020). Vaccination – a step closer to universal health coverage. *Journal of Public Health* 30, 649–653. doi: 10.1007/s10389-020-01322-y

Kamwi, R.N. (2016) Gene drive debate must include voices from Africa, *elsewhere. STAT News*, 15 June 2016. Available at: https://www.statnews.com/2016/06/15/gene-drive-debate-africa/ (accessed April 2022)

Katz, E. (2000) The big lie. The human restoration of nature. In: Throop, W. (ed.) *Environmental Restoration: Ethics, Theory, and Practice*. Humanity Books, New York, pp. 83–93.

Kelsch, A., Takahashi, Y., Dasgupta, R., Mader, A.D., Johnson, B.A. and Kumar, P. (2020) Invasive alien species and local communities in socio-ecological production landscapes and seascapes: a systematic review and analysis. *Environmental Science and Policy*, 112, 275–281. doi: 10.1016/j.envsci.2020.06.014

Kokotovich, A.E., Delborne, J.A., Elsensohn, J. and Burrack, H. (2020) Emerging technologies for invasive insects: the role of engagement. *Annals of the Entomological Society of America* 113, 266–279. doi: 10.1093/aesa/saz064

Kolopack, P.A. and Lavery, J.V. (2017) Informed consent in field trials of gene-drive mosquitoes. *Gates Open Research* 1(0), 14. doi: 10.12688/gatesopenres.12771.1

Kolopack, P.A., Parsons, J.A. and Lavery, J.V. (2015) What makes community engagement effective? Lessons from the Eliminate Dengue Program in Queensland Australia. *PLoS Neglected Tropical Diseases* 9(4), 1–19. doi: 10.1371/journal.pntd.0003713

Kormos, A., Lanzaro, G.C., Bier, E., Dimopoulos, G., Marshall, J.M. *et al.* (2021) Application of the relationship-based model to engagement for field trials of genetically engineered malaria vectors. *American Journal of Tropical Medicine and Hygiene* 104(3), 805–811. doi: 10.4269/ajtmh.20-0868

Kraemer, F. (2010) Emotions involved in risk perception: from sociological and psychological risk studies towards a neosentimentalist meta-ethics. In: Roeser, S. (ed.) *Emotions and Risky Technologies*. Springer, Dordrecht, Netherlands. pp. 195–211. doi: 10.1007/978-90-481-8647-1_12

Lavery, J.V., Tinadana, P.O., Scott, T.W., Harrington, L.C., Ramsey, J.M. *et al.* (2010) Towards a framework for community engagement in global health research. *Trends in Parasitology* 26(6), 279–283. doi: 10.1016/j.pt.2010.02.009

Ledingham, K. and Hartley, S. (2021) Transformation and slippage in co-production ambitions for global technology development: the case of gene drive. *Environmental Science and Policy* 116, 78–85. doi: 10.1016/j.envsci.2020.10.014

Mankad, A., Hobman, E. and Carter, L. (2020) Effects of knowledge and emotion on support for novel synthetic biology applications. *Conservation Biology* 35, 623–633. doi: 10.1111/cobi.13637

Marris, C. (2015) The construction of imaginaries of the public as a threat to synthetic biology. *Science as Culture* 24(1), 83–98. doi: 10.1080/09505431.2014.986320

Mazerik, J. and Rejeski, D. (2014) *A Guide for Communicating Synthetic Biology*. Synthetic Biology Project, Wilson Center, Washington, DC.

Michener, L., Cook, J., Ahmed, S.M., Yonas, M.A., Coyne-Beasley, T. and Aguilar-Gaxiola, S. (2012) Aligning the goals of community-engaged research: why and how academic health centers can successfully engage with communities to improve health. *Academic Medicine* 87(3), 285–291. doi: 10.1097/ACM.0b013e3182441680

Nadal, C., Sas, C. and Doherty, G. (2019) Technology acceptability, acceptance and adoption - definitions and measurement. Paper presented at 2019 *CHI Conference on Human Factors in Computing Systems*, Glasgow, UK.

NASEM (2016) *Gene Drives on the Horizon*. National Academies of Sciences Engineering and Medicine, Washington, DC. doi: 10.17226/23405

Newson, A. (2015) Synthetic biology: ethics, exceptionalism and expectations. *Macquarie Law Journal*, 15 (May 2013), 45–58. Available at: http://content.ebscohost.com/ContentServer.asp?T=P&P=AN&K=10 8715935&S=R&D=ofs&EbscoContent=dGJyMNLe80SeprM4zdnyOLCmr0%2BeqK9Sr664Sa%2B-WxWXS&ContentCustomer=dGJyMPGpt02xr65LuePfgeyx43zx

North, A.R., Burt, A. and Godfray, H.C.J. (2019) Modelling the potential of genetic control of malaria mosquitoes at national scale. *BMC Biology* 17(1), 26. doi: 10.1186/s12915-019-0645-5

Palmer, C. (2002) An overview of environmental ethics. In: Light, A. and Rolston, H. III (eds) *Environmental Ethics: An Anthology*. Wiley-Blackwell, Oxford, UK, pp. 15–37. https://www.wiley.com/en-us/Environmental+Ethics%3A+An+Anthology-p-9780631222941

Pare Toe, L., Dicko, B., Linga, R., Barry, N., Drabo, M. *et al.* (2021) Operationalising stakeholder engagement for gene drive research in malaria elimination in Africa – balancing local context specificities and international recommendations. Nature Communications [in review].

Pielke, R. Jr (2007) *The Honest Broker: Making Sense of Science in Policy and Politics*. Cambridge University Press, Cambridge. doi: 10.1017/CBO9780511818110

Preston, C.J. (2008) Synthetic biology: drawing a line in Darwin's sand. *Environmental Values* 17(1). doi: 10.3197/096327108X271932

Pulford, J., Hetzel, M.W., Bryant, M., Siba, P.M. and Mueller, I. (2011) Reported reasons for not using a mosquito net when one is available: a review of the published literature. *Malaria Journal* 10(1), 83. doi: 10.1186/1475-2875-10-83

Ramsey, J.M., Bond, J.G., Macotela, M.E., Facchinelli, L., Valerio, L. *et al.* (2014). A regulatory structure for working with genetically modified mosquitoes: lessons from Mexico. *PLoS Neglected Tropical Diseases* 8(3). doi: 10.1371/journal.pntd.0002623

Redford, K.H., Brooks, T.M., Nicholas, B.W. and Adams, J.S. (2019) *Genetic Frontiers for Conservation: an assessment of synthetic biology and biodiversity conservation: technical assessment*. IUCN, Gland, Switzerland. doi: 10.2305/iucn.ch.2019.05.en

Reed, M.S. and Curzon, R. (2015) Stakeholder mapping for the governance of biosecurity: a literature review. *Journal of Integrative Environmental Sciences* 12(1). doi: 10.1080/1943815X.2014.975723

Reynolds, J.L. (2021) Engineering biological diversity: the international governance of synthetic biology, gene drives, and de-extinction for conservation. *Current Opinion in Environmental Sustainability*, 49, 1–6. doi: 10.1016/j.cosust.2020.10.001

Reynolds, L. and Sariola, S. (2018) The ethics and politics of community engagement in global health research. *Critical Public Health* 28(3). doi: 10.1080/09581596.2018.1449598

Roberts, A. and Thizy, D. (2021) Ethical principles informing Target Malaria's engagement strategy: timely co-development with relevant communities. *Malaria Journal* [in review]

Rogers, E. (2004) A prospective and retrospective look at the diffusion model. *Journal of Health Communication* 9 (Suppl. 1), 13–19. doi: 10.1080/10810730490271449

Rohwer, Y. and Marris, E. (2021) Ecosystem integrity is neither real nor valuable. *Conservation Science and Practice* 3(4), e411. doi: 10.1111/csp2.411

Rolston, H.I. (1985) Duties to endangered species. *BioScience* 35(11), 718–726. doi: 10.2307/1310053

Rolston, H.I. (1989) The value of species. In: Regan, T. and Singer, P. (eds) *Animal Rights and Human Obligations*, 2nd edn. Cambridge University Press, Cambridge.

Sarewitz, D. (2004) How science makes environmental controversies worse. *Environmental Science & Policy* 7(5), 385–403. doi: 10.1016/j.envsci.2004.06.001

Savary, S., Willocquet, L., Pethybridge, S.J., Esker, P., McRoberts, N. and Nelson, A. (2019) The global burden of pathogens and pests on major food crops. *Nature Ecology & Evolution* 3(3), 430–439. doi: 10.1038/s41559-018-0793-y

Schwarz, N. and Clore, G. (1983) Mood, misattribution, and judgments of well-being: informative and directive functions of affective states. *Journal of Personality and Social Psychology* 45, 513–523. doi: 10.1037/0022-3514.45.3.513

Singh, J.A. (2019) Informed consent and community engagement in open field research: lessons for gene drive science. *BMC Medical Ethics* 20(1), 1–12. doi: 10.1186/s12910-019-0389-3

Slovic, P. (1999) Trust, emotion, sex, politics, and science: surveying the risk-assessment battlefield. *Risk Analysis* 19(4), 689–701. doi: 10.1023/A:1007041821623

Slovic, P., Finucane, M.L., Peters, E. and MacGregor, D.G. (2004) Risk as analysis and risk as feelings: some thoughts about affect, reason, risk, and rationality. *Risk Analysis* 24(2), 311–322. doi: 10.1111/j.0272-4332.2004.00433.x

Stirling, A., Hayes, K.R. and Delborne, J. (2018) Towards inclusive social appraisal: risk, participation and democracy in governance of synthetic biology. *BMC Proceedings* 12 (Suppl. 8), 15. doi: 10.1186/s12919-018-0111-3

Sturgis, P. and Allum, N. (2004) Science in society: re-evaluating the deficit model of public attitudes. *Public Understanding of Science* 13(1), 55–74. doi: 10.1177/0963662504042690

Syme, G.J. and Nancarrow, B.E, (1991) Risk perception and procedural justice. In: Handmer, J., Dutton, B., Guerin, B. and Smithson, M. (eds) *New Perspectives on Uncertainty and Risk*. Centre for Resource and Environmental Studies and the Australian Counter Disaster College, Canberra, pp. 87–94.

Teisl, M.F., Fein, S.B. and Levy, A.S. (2009) Information effects on consumer attitudes toward three food technologies: organic production, biotechnology, and irradiation. *Food Quality and Preference* 20(8), 586–596. doi: 10.1016/j.foodqual.2009.07.001

Thibaut, J.W. and Walker, L. (1975) *Procedural Justice: a Psychological Analysis*. Lawrence Earlbaum Associates, Hillsdale, New Jersey.

Thizy, D., Emerson, C., Gibbs, J., Hartley, S., Kapiriri, L. *et al.* (2019) Guidance on stakeholder engagement practices to inform the development of area-wide vector control methods. *PLoS Neglected Tropical Diseases* 13(4), e.0007286. doi: 10.1371/journal.pntd.0007286

Thizy, D., Coche, I. and de Vries, J. (2020) Providing a policy framework for responsible gene drive research: an analysis of the existing governance landscape and priority areas for further research. *Wellcome Open Research* 5, 173. doi: 10.12688/wellcomeopenres.16023.1

Thizy, D, Pare Toe, L., Mbogo, C., Matoke-Muhia, D., Alibu, V.P. *et al.* (2021) Proceedings of an expert workshop on community agreement for gene drive research in Africa – Co-organised by KEMRI, PAMCA and Target Malaria. *Gates Open Research* 5(19). doi: 10.12688/gatesopenres.13221.2

Tokponnon, F.T., Sissinto, Y., Ogouyémi, A.H., Adéothy, A.A., Adechoubou, A. *et al.* (2019) Implications of insecticide resistance for malaria vector control with long-lasting insecticidal nets: Evidence from health facility data from Benin. *Malaria Journal* 18(1), 37. doi: 10.1186/s12936-019-2656-7

Townson, H. (2009) SIT for African malaria vectors: Epilogue. *Malaria Journal* 8(2), S10. doi: 10.1186/1475-2875-8-S2-S10

Vogel, S. (2015) *Thinking Like a Mall: Environmental Philosophy after the End of Nature*. MIT Press, Cambridge, Massachusetts. doi: 10.5840/enviroethics201638442

Wanyama Chemonges, E., Pare Toe, L., Dicko, B. and Coulibaly, M. (2021) Co-developing a common glossary with stakeholders for engagement on new genetic approaches for malaria control in a local African setting. *Malaria Journal* 20, Article 53.

Wassilak, S. and Orenstein, W. (2010) Challenges faced by the global polio eradication initiative. *Expert Review of Vaccines* 9(5), 447–449. doi: 10.1586/erv.10.45

Waytz, A. and Young, L. (2019) Aversion to playing God and moral condemnation of technology and science. *Philosophical Transactions of the Royal Society B: Biological Sciences* 374(1771), 20180041. doi: 10.1098/rstb.2018.0041

WHO (2012) Standards and operational guidance for ethics review of health-related research with human participants. *Acta Bioethica* 18(1), 129–132. doi: 10.4067/s1726-569x2012000100014

WHO (2016) *Global report on insecticide resistance in malaria vectors: 2010–2016*. World Health Organization, Geneva. Available at: http://www.who.int/malaria/publications/atoz/9789241514057/en/ (accessed April 2022)

WHO (2017a) *The evaluation process for vector control products*. Information Note. World Health Organization, Geneva.

WHO (2017b) *Global Vector Control Response 2017–2030*. World Health Organization, Geneva. *Available at*: https://apps.who.int/iris/bitstream/handle/10665/259205/9789241512978-eng.pdf?sequence=1 (accessed April 2022)

WHO (2019) *Malaria eradication: benefits, future scenarios and feasibility*. Executive summary. A report of the Strategic Advisory Group on Malaria Eradication. World Health Organization, Geneva. Available at: https://www.who.int/publications-detail/strategic-advisory-group-malaria-eradication-executive-summary (accessed April 2022)

WHO (2020a) *Evaluation of genetically modified mosquitoes for the control of vector-borne diseases*. Position Statement (Issue October). World Health Organization Geneva. Available at: https://www.who.int/publications/i/item/9789240013155 (accessed April 2022)

WHO (2020b) *Ethics and vector borne diseases*. WHO Guidance. World Health Organization, Geneva. Available at: https://apps.who.int/iris/bitstream/handle/10665/336075/9789240012738-eng.pdf (accessed April 2022)

WHO (2021a) *Guidance Framework for Testing Genetically Modified Mosquitoes*, 2nd edn. World Health Organization, Geneva. Available at: https://www.who.int/publications/i/item/9789240025233 (accessed April 2022)

WHO (2021b) *Updating the WHO Global technical strategy for malaria 2016–2030*. World Health Organization, Geneva. Available at: https://www.who.int/news-room/events/detail/2021/01/28/default-calendar/updating-the-who-global-technical-strategy-for-malaria-2016-2030 (accessed April 2022)

WHO/TDR (2014) *Guidance Framework for testing genetically modified mosquitoes*. World Health Organization / Special Programme for Research and Training in Tropical Diseases (TDR), Geneva. Available at: http://r4d.dfid.gov.uk/Output/197475/Default.aspx and https://apps.who.int/iris/handle/10665/127889 (accessed April 2022)

Winner, L. (1980) Do artifacts have politics? *Daedalus* 109(1), 121–136. Available at: http://www.jstor.org/stable/20024652 (accessed April 2022)

25 Regulation of Transgenic Insects

Camilla Beech[1]*, Nathan Rose[2] and Brinda Dass[3]

[1]*Cambea Consulting Ltd, Reading, UK;* [2]*Oxitec Ltd, 71 Innovation Drive, Milton Park, Abingdon, UK;* [3]*Foundation for the National Institutes of Health, 11400 Rockville Pike, North Bethesda, Maryland, USA.*

25.1 Introduction

Pest insects are one of the leading causes of significant economic damage and harm to humans. Insects can transmit human, animal and plant diseases, as well as directly attacking crops and animals, causing both damage and yield losses. International quality standards for agricultural and horticultural crops (such as the World Trade Organization Agreement on Sanitary and Phytosanitary Standards (WTO SPS)) mean that pest damage can also limit trade. Bradshaw *et al*. (2016) estimated that, based on figures from 2014, invasive insects cost a minimum of US$70.0 billion per year globally, with associated health costs exceeding US$6.9 billion per year. Furthermore, the expected impact of climate change is likely to exacerbate losses in agriculture and human/animal health further (Pureswaran *et al*., 2018; Botha *et al*., 2019; Giesen *et al*., 2020; Lehmann *et al*., 2020). Efforts to control insect pests have largely relied on chemical control methods; however, resistance to these is increasing, along with lower societal acceptance of pesticide residues, both in food and in the environment.

In the case of human diseases, mosquitoes are the major pests of concern. Mosquitoes transmit malaria parasites and viral diseases such as dengue fever, which is recognized as the world's major emerging tropical disease with 5.2 million cases reported to the World Health Organization in 2019 (WHO, 2021a), although this is considered an underestimate and cases could be as many as 390 million annually (Bhatt *et al*., 2013). Malaria is reported (WHO, 2020) to have had an estimated 229 million cases in 2019 in 87 malaria-endemic countries, with the African region accounting for 94% of cases. Malaria mortality is common in children under 5 years of age, amounting to 67% of total malaria deaths in 2019. Both of these mosquito-borne diseases cause widespread mortality and morbidity, as well as socio-economic burdens such as loss of productivity, family disruption, school absenteeism and additional health-related out-of-pocket expenditure.

At present the human population has limited defences against mosquitoes that transmit diseases, aside from a small number of chemical and biological pesticides, insecticide-treated bednets and screens on

---

*Corresponding author, email: cambeaconsults@gmail.com

windows and doors, and breeding site reductions, although several new chemical delivery mechanisms are in development, such as Eave Tubes, Spatial Repellents and Attractive Toxic Sugar Bait stations (WHO, 2021c). Very recently a vaccine candidate for malaria has been reported as providing 75% efficacy (Datoo *et al*., 2021; Mahase, 2021) and recommended for children in sub-Saharan Africa by WHO (Chandramohan *et al*., 2021; WHO, 2021b); however, universal coverage may remain many years away. Furthermore, physical defences, such as bednets and house screens, are ineffective for a daytime-biting mosquito such as *Aedes aegypti* that is well adapted to cohabiting with humans, and which is the major vector of dengue fever.

Ae. aegypti has pan-tropical distribution and is an invasive species in countries outside Africa. Southeast Asia and South America are the worst affected by *Aedes*-borne diseases. Brazil has the highest number of dengue cases and economic burden from dengue, estimated at over 2 million cases and US$900 million per year in 2010 without prevention costs (Shepard *et al*., 2011); vector control costs alone are over US$500 million (Barreto *et al*., 2011). While this in part reflects the size of the country, Medlock *et al*. (2009) concluded that the dengue burden was as high as the burden of other major infectious diseases that afflict the Brazilian population, including malaria. The recent Zika virus epidemic in 2015–2016 led WHO to declare a Public Health Emergency of International Concern in 2016, due to the links between Zika infection and microcephaly in infants. Brazil has also suffered chikungunya outbreaks, with over 700,000 cases reported in a four-year period between 2011 and 2015 (Cunha *et al*., 2020). Outbreaks of yellow fever were also reported in Brazil in 2017, which was followed by mass vaccination campaigns to contain the spread of disease (Zanotto and Leite, 2018).

Meanwhile, the only approved dengue vaccine, Dengvaxia (CYD-TDV), has limited utility; while clinical trials demonstrated that it was protective in 76% of trial participants already seropositive for dengue, it was far less effective in trial participants seronegative for dengue, with increased risks of hospitalization and severe dengue in seronegative individuals. As a result, the vaccine is only recommended for use in seropositive individuals (WHO, 2018).

Consequently, new vector control methods are urgently required for mosquito-to-borne diseases. Several genetics-based insect control strategies have received national commercial biosafety approval in Brazil or are under evaluation by national regulatory authorities in other countries. In Brazil, the private company Oxitec Ltd has received commercial biosafety approval for two genetically modified (GM) *Ae. aegypti* strains: OX513A and OX5034. OX513A, which carries a self-limiting gene that causes both male and female mortality, required mechanical separation of male insects prior to release (see Morrison, Chapter 23, this volume). OX5034, a genetic sexing strain, carries a self-limiting gene that causes only female larvicidal mortality, leaving male mosquitoes unaffected. Both have been released extensively in Brazil, with over 1 billion modified *Ae. aegypti* released to date, and effective field performance has been demonstrated in multiple countries (Phuc *et al*., 2007; Harris *et al*., 2012; Carvalho *et al*., 2015; Gorman *et al*., 2016). The development of genetically modified malaria vectors is also in advanced stages, with the first experimental field releases of sterile male *Anopheles coluzzii,* a member of the *Anopheles gambiae* s.l. species complex, occurring in Burkina Faso in 2019 (Pare Toe *et al*., 2021).

Similar approaches are also in advanced development for a range of crop pests. Self-limiting insects have been developed by Oxitec Ltd to target fall armyworm, diamondback moth, pink bollworm, Mediterranean fruit fly, olive fly and others (see Scott *et al*., Chapter 17, this volume). Other developers have focused on the important soft fruit pest, spotted-wing *Drosophila suzukii* (Li *et al*., 2021). Genetic sexing strains of screwworm and blowfly have also been developed (Concha *et al*., 2016; Yan and Scott, 2020). Oxitec carried out open field releases of diamondback moth in New York state, USA, in 2017 (Shelton *et al*., 2020) and fall armyworm in Sao Paulo state in Brazil in 2019–2020 (Oxitec, 2021) as well as caged

crop protection trials of Mediterranean fruit fly in Morocco (Asadi *et al.*, 2019).

Insects are also emerging as a new sector for the food, feed and industrial products industries (van Huis, 2020) to provide ingredients for consumer products, with mealworms (*Tenebrio molitor*) and black soldier fly (*Hermetia illucens*) predominating for food and feed, and silkworms (*Bombyx mori*) for industrial textiles. While these early food and feed products are currently using classical genetics to improve yields and other characteristics, there is ambition in the field to use genetic modification in the future (Zhan *et al.*, 2020). Silkworm transgenesis for industrial products has been established for many years (Kuwana *et al.*, 2014) (see Sezutsu and Tamura, Chapter 20, this volume).

There has also been a surge of scientific advances for genetic engineering of insects with the advent of new tools and techniques such as gene editing based on clustered regularly interspaced short palindromic repeats (CRISPR) (Jinek *et al.*, 2012), offering the potential to introduce genetic elements that bias genetic inheritance so that introduced traits are preferentially inherited even when there is the potential for negative fitness consequences – a phenomenon known as gene drive (Burt and Crisanti, 2018). Gene drive constructs in insects can result in a spectrum of potential phenotypes and cannot be assessed generically as a class but on a case-by-case basis. These scientific advances are moving quickly and will only continue to improve in efficiency and pace.

This chapter will focus on governance and regulatory frameworks, regulatory coordination and harmonization, including common themes in regulatory requirements, for the use of GM insects (including those with gene drives), the current regulatory status of GM insects, and considerations regarding the use of public participation in decision making.

25.2 Governance and Regulatory Frameworks

The governance of genetically modified organisms (GMOs) has sometimes been

controversial, and, as mentioned in the first edition of this chapter, early interventions against GMO crops set the tone for governance of GM insects, including legal challenge of governmental decision making, usually based on process and legal technicalities rather than on scientific grounds. Debates around GM insects are being played out in the same intergovernmental forums (relating to the Convention on Biological Diversity); they have similar advocacy and activist groups to those for GM crops, and similar interpretations of the precautionary principle. This non-neutral background sets the context for the governance and regulation of GM insects, including those with engineered gene drives.

A number of international treaties affect the global governance of GMOs, including those with engineered gene drives. The primary treaty is the Convention on Biological Diversity (CBD) and its associated Protocols. The Cartagena Protocol on Biosafety (CPB) to the Convention on Biological Diversity (CBD, 2018a) provides an international framework for the safe transfer, handling and use of GMOs (defined as living modified organisms (LMOs) in the definitions of the Protocol), including GM insects and gene drives in insects (see Pereira, Chapter 27, this volume).

The CPB enshrines the 'precautionary principle' defined in Principle 15 in the Rio Declaration on Biodiversity (UN, 1992):

> In order to protect the environment, the precautionary approach shall be widely applied by States according to their capabilities. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation.

Ideally, interpretation and use of the precautionary principle should be about finding the correct balance to make proportionate, evidence-based decisions that provide an appropriate level of protection; a structure that is well framed in the three elements of risk analysis: risk assessment, risk management and risk communication. Frequently, however, the precautionary principle is

incorrectly interpreted, with the aim of preventing any action, the implications of which have been reviewed (Bhattacharya, 2002; Cantley, 2012; Guidotti, 2012; CAST, 2013). However, it has also been interpreted by the European Commission itself (EC, 2000) as adherence to the general principles of risk management, which include: (i) the principle of proportionality between the measures taken and the chosen level of protection; (ii) the principle of non-discrimination in the application of measures; (iii) the consistency of the measures with similar measures already taken for similar situations; (iv) the examination of benefits and costs of action or inaction; and (v) review of measures in the light of scientific developments.

The CPB was finalized and adopted in January 2000 and entered into force in September 2003. As of July 2020, there were 173 Parties to the CPB. The CPB acquires force at a national level through implementations in the Parties' legal systems. These proceed asynchronously, as each country takes time to develop or adapt law through its national governmental processes. Many Parties, especially those with transitional economies, received initial support to develop and implement national biosafety frameworks, implementing regulations, organizational structures and capacity to assess products of modern biotechnology in response to the CPB (UNEP, 2005; McLean *et al*., 2012). Notable non-Parties to the CPB (or Parties that have not yet ratified the CPB) include Argentina, Australia, Canada, Chile, Israel, Singapore and the USA, although most of these countries have their own domestic regulatory frameworks for GMOs. Subsequent to the implementation of the CPB, several Parties have carried out regulatory risk assessments for transboundary movements and intentional releases of GM insects into the environment (Table 25.1).

Governance, particularly with respect to the use of organisms engineered to contain gene drives for public health, agriculture and conservation biology, is under significant debate within the CBD at the time of writing. A decision on the CPB (in November 2018) from the 9th meeting of the Conference of the Parties/Meeting of the Parties (to the CPB) (COP-MOP) outlined Parties' interests in identifying and prioritizing specific issues related to the risk assessment of living modified organisms (LMOs) and utilizing an online forum as well as a newly constituted *ad hoc* technical group of experts (AHTEG) to consider the need for developing further guidance on risk assessment of LMOs containing engineered gene drives (EGDOs). The 24th Subsidiary Body on Scientific, Technical and Technological Advice (SBSTTA) was tasked with providing a recommendation on the need for additional guidance material on risk assessment of EGDOs for consideration by Parties at the 10th COP-MOP on the CPB (CBD, 2018b). The AHTEG agreed that LMOs with engineered or synthetic gene drives were within the scope of CPB, that existing risk assessment methodology is applicable to EGDOs but challenged by limitations in available data, including effects on ecosystems, monitoring and surveillance, and validated modelling tools, spread and persistence of EGDOs, lack of reversibility methods and lack of experience and capacity. The AHTEG recommended that guidance for EGDOs be developed (AHTEG, 2020). This decision will be considered by the SBSTTA, who will determine whether to forward it to COP-MOP for discussion by all Parties.

In the field of GM insects, WHO and its Special Programme for Research and Training in Tropical Diseases (TDR) has historically taken a lead in considering the issues raised by the genetic modification of insects that are vectors of human disease (WHO/TDR, 1991), by hosting international expert consultations and other fora (Takken *et al*., 2002; Knols and Bossin, 2006; WHO, 2010; Beech *et al*., 2011), and particularly through capacity building. WHO has continued to lead in this area by initiating development of the *Guidance Framework for Testing of Genetically Modified Mosquitoes*. This document was published in 2014 (WHO/TDR and FNIH, 2014) and updated in 2021 (WHO/TDR, 2021), which recommended a stepwise testing pathway for GM mosquitoes as public health tools. The pathway had three main stages: testing in a physically confined laboratory or insectary setting, then a physically or

Table 25.1. Genetically modified insects that have been released into the environment.

Insect species	Strain details	Countries	Release purpose	Company/ organization
Aedes aegypti	OX513A (self-limiting strain)	Cayman Islands (2010–2018) Brazil (2010–2014) Panama (2014) Malaysia (2010)	Field trials (self-limiting strain)	Oxitec Ltd
		Brazil (2014–2018)	Post-commercial approval release	Oxitec Ltd
	OX5034 (female-specific self-limiting strain)	Brazil (2018–2020) USA (2021–)	Field trials	Oxitec Ltd
		Brazil (2020–)	Post-commercial approval release	Oxitec Ltd
Anopheles gambiae	Ac(DSM)2 (sterile male strain)	Burkina Faso (2019)	Field trials	Institut de Recherche en Sciences de la Santé/Target Malaria
Pectinophora gossypiella (pink bollworm)	OX1138B (marker-only strain)	USA (2006–2007)	Field trials	Oxitec Ltd
Plutella xylostella (diamondback moth)	OX4319L (female-specific self-limiting strain)	USA (2017)	Field trials	Oxitec Ltd
Spodoptera frugiperda (fall armyworm)	OX5382G (female-specific self-limiting strain)	Brazil (2019–2021)	Field trials	Oxitec Ltd
		Brazil (2021–)	Post-commercial approval release	Oxitec Ltd

ecologically confined field testing, before moving to field releases of increasing scale and complexity. The document also described the elements required for evaluation of product safety, quality and efficacy, with a clear go/no-go decision criterion based on the criterion that the GM mosquito 'will do no more harm to human health than wild-type mosquitoes of the same genetic background and no more harm to the ecosystem than other conventional vector control interventions'. The guidance framework is envisaged as a living document to be updated as necessary to keep pace with research on GM mosquitoes.

WHO also has an evidence-based framework for the independent evaluation of all new vector control technologies and approaches, including genetic-based technologies (WHO, 2017). WHO evaluates the safety, efficacy, quality and public health value of new vector control tools, via a single entry point managed by the WHO prequalification team for vector control (PQT-VC). A pre-submission coordination committee determines if the product has potential for use in disease control programmes and whether it falls within an established or new intervention product class. The new intervention pathway relies on assessment and advice from the Vector Control Advisory Group (VCAG). VCAG evaluates the products for public health value based on epidemiological evidence, supported by relevant entomological evidence from well-controlled studies. VCAG works in concert with PQT-VC. If a positive recommendation is provided by VCAG there is further scrutiny within WHO by the relevant policy committee (the Malaria Policy Advisory Group (MPAG) for malaria and the Strategic and Technical Advisory Group (STAG) for neglected tropical diseases). If endorsed by either policy committee, then WHO issues a policy recommendation and operational guidance. PQT-VC then manages post-qualification activities for the product.

In October 2020, WHO issued two position statements recognizing the urgent need for new tools to combat vector-borne diseases and that all potential technologies should be investigated; the first on the 'Evaluation of Genetically Modified Mosquitoes for the Control of Vector Borne Diseases' and secondly on 'Ethics and Vector Borne Diseases'. The statement on the evaluation supports the stepwise approach of evaluation of GM mosquitoes, together with clear governance mechanisms to evaluate potential impacts on human health and the environment, including the engagement of communities for area-wide control measures. It also states that 'current governance mechanisms should be adapted to purpose rather than replaced' and that 'internationally recognised risk assessment tools and procedures should be used for the evaluation of safety, with account taken of potential health benefits'. This focus on potential health benefits, alongside the assessment of potential risks, is an important development in the evaluation of these new vector control tools.

25.2.1   Regulatory frameworks

The purpose of regulation is to provide an assurance of safety of a product prior to entry into the marketplace, as well as compliance with the applicable national, local and other relevant laws once in commercial or general use. This is particularly important, as national, local and other relevant laws ideally derive from the values of the society at large and thereby define common protection goals at the highest level. Thus, regulations determine the scope of the determinations of safety being made for each product. Regulation also provides a common basis for making these determinations for all products of a particular type or class. This allows transparency and predictability of expectations and criteria for both product developers and independent assessors.

GM insects are subject to regulation in countries that are Parties to the Cartagena Protocol on Biosafety (CPB), whose definition of living modified organisms (LMOs) includes these insects (CBD, 2000). As mentioned above, many Parties to the CPB have made substantial progress in developing

and implementing their biosafety systems, including regulations and organizational structures aimed at building their capacity to regulate LMOs. However, only a limited number have sufficiently operationalized their systems to be considered functional to adequately assess and allow testing and use of LMOs, especially GM insects. Many of these countries that have operational capacity and experience with assessment and regulation of GMOs have considered GM crop applications, but not GM insects or other GM animals. Countries that have permitted the use of GM mosquitoes for inundative releases to control vector species such as *Ae. aegypti* and plant pest species such as fall armyworm (*Spodoptera frugiperda*) currently have the most experience in regulating GM insects (Table 25.1).

Early adopters of GM technology, such as the USA and Canada, developed their regulatory systems in response to advances in technology, and not in response to the CPB, to which they are not Parties (CBD, 2018a). In the USA, the Office of Science and Technology Policy (OSTP) determined that existing legislation was sufficient to regulate and ensure safety of products of recombinant DNA technology and outlined the comprehensive federal regulatory policy in the Coordinated Framework for the Regulation of Biotechnology which was developed in 1986 (OSTP, 1986), updated in 1992 (OSTP, 1992) and 2017 (OSTP, 2017). The three primary US Federal agencies with oversight of biotechnology products are the US Environmental Protection Agency (EPA), US Food and Drug Administration (FDA) and US Department of Agriculture (USDA) (USDA, 2021). Regulation of GM insects in the USA has had the clearest path with GM plant-pest insect species, which have been evaluated by USDA (e.g. pink bollworm moth (*Pectinophora gossypiella*) (USDA APHIS, 2006, 2008, 2009; Simmons *et al.*, 2011), Mediterranean fruit fly (*Ceratitis capitata*), Mexican fruit fly (*Anastrepha ludens*), oriental fruit fly (*Bactrocera dorsalis*) (USDA APHIS, 2008, 2009); and diamondback moth (*Plutella xylostella*) (Shelton *et al.*, 2020)) under the Plant Protection Act (PPA).

Jurisdiction has been less clear for GM disease vector species such as *Ae. aegypti*, which could be regulated by FDA as a new animal drug (human or animal disease prevention and pathogen load reduction) or EPA as a pesticide (insect population suppression), depending on the intended use (FDA-CVM, 2017). Import of species that are known vectors of human diseases may also require an import permit from the US Centers for Disease Control and Prevention (CDC, 2021).

Brazil has also been an early adopter of GM technology and is the first country to grant commercial biosafety approval to GM insects. In Brazil, all aspects of biosafety for GMOs (plants, animals and micro-organisms) are regulated by the National Technical Biosafety Commission (CTNBio). Under the terms of Brazil's 2005 Biosafety Law, CTNBio's decisions on biosafety are binding on other government agencies and ministries, such as the Ministry of Agriculture, the Health Surveillance Agency (ANVISA) and the Ministry of the Environment. Brazil has granted commercial biosafety approval to three GM insect strains developed by Oxitec Ltd, including the two *Ae. aegypti* strains OX513A (in 2014) and OX5034 (in 2020), and the fall armyworm (*S. frugiperda*) strain OX5382G (in 2021).

Regulatory systems in Sub-Saharan Africa (SSA), the likely setting for future releases of GM *Anopheles* mosquitoes for malaria vector control, are at different stages of development (Ecuru, 2017; Komen *et al.*, 2020). Some are well advanced; for example, Nigeria has had the foresight to include synthetic biology and gene drives in their regulations, while others have yet to put functional regulatory systems in place. The focus remains on capacity building, with the institutional resources required to undertake regulatory review and enforcement a key consideration (Obonyo *et al.*, 2011). However, the development of functional regulatory systems may also relate to the absence of applications to assess – though the absence of operational ability to regulate itself inhibits applications in a cycle of inadequate capacity.

25.2.2 Coordination of efforts for GM insect regulation

Coordination, especially regional harmonization, could ease regulation, in terms of providing a one-stop-shop approach, with uniformity of requirements, risk assessment, risk mitigation, ease of cross-border transport and trade, monitoring and surveillance, as well as immediate benefits in terms of capacity building, common data packages and mutual recognition of efficacy data and field trials (Minde and Mazvimavi, 2007). Given the natural ability of insects to disperse, regional regulatory systems could facilitate compliance with transnational regulatory systems such as those under CBD and allied protocols. This could take several forms, including subregional/regional or international regulatory harmonization that recognizes scientific assessments from other regulatory agencies, data produced in other countries or even acceptance of decisions taken by other governments.

The Organisation for Economic Co-operation and Development (OECD) supports activities and networks in the field of biotechnology through a Working Group on Harmonisation of Regulatory Oversight of Biotechnology, to improve mutual understanding and harmonized practise in biosafety evaluation. The working group has published 68 technical consensus documents on specific aspects of biotechnology at a joint governmental level to support national processes for risk assessment. This group has issued a consensus document on the biology of *Ae. aegypti* (OECD, 2018) and is currently preparing one on the biology of *Anopheles gambiae* (OECD, 2021). Coordination of regulatory requirements for GM insects has been carried out in the past in some areas; for example: the North American Plant Protection Organization (NAPPO), representing the USA, Mexico and Canada, Standard on GE Arthropods RSPM 27; the Framework Convention on Common Biosafety Regulations in CILSS Countries (representing nine African nations); and the Iniciativa Regional en Biotecnología y Bioseguridad para América Central, which represents seven Central American nations.

In 2017 the African Union (AU) High-Level Panel on Emerging Technologies (APET) recommended that gene drives be thoroughly examined as a realistic option for effective malaria control and elimination as part of existing integrated vector management (IVM) (AUDA-NEPAD, 2018). Following this, the Assembly of African ministers of Science and Technology (AU, 2017) and the AU Executive Council at its 32nd Ordinary Session (AU, 2018) also recommended that AU member states should consider gene drive insects in their development plans. The African Union Development Agency–New Partnership for Africa's Development (AUDA-NEPAD) has worked with the West Africa Health Organization (WAHO) to establish an AU-recognized, regional IVM platform aligned with ECOWAS member states to implement these AU resolutions on IVM. The resulting West Africa IVM platform (WA-IVM), with its inaugural meeting in April 2019 (AUDA-NEPAD, 2020), includes health and environment regulators, ethics committee members and malaria control programme managers from Burkina Faso, Côte d'Ivoire, Ghana, Mali, Nigeria and Senegal in a One Health, multisectoral regional governance approach for vector control and elimination. A regional steering committee comprising ECOWAS heads of Agriculture, Environment/Biosafety and national medicines regulators will obtain expert opinions from four technical working groups – Health, Biosafety, Disease Management and Vector Control, and Ethics – on which to base their recommendations to relevant ECOWAS ministers and heads of state. Decision making will be at the level of member states. Other attempts at regional decision making on GMOs, such as the Common Market for Eastern and Southern Africa (COMESA)'s Regional Approach to Biotechnology and Biosafety Policy in Eastern and Southern Africa (RABESA) initiative, driven by CP Article 14 and COMESA Treaty Articles 129 and 130(a), have developed a regional Policy on biotechnology and biosafety (Waithaka *et al*., 2015). The Policy is not a legal instrument and does not require domestication, allowing countries to retain sovereignty over decision making. The 5-year (2014–2019) COMESA Biotechnology and Biosafety Policy Implementation

Plan (COMBIP) that followed was to implement the regional Policy and create a regional biosafety risk assessment mechanism (COMESA, 2019).

National regulations also govern the conditions under which non-GM insects, such as beneficial insects or sterile insect technique (SIT) releases can be used. In both examples large quantities of insects are mass-reared and released into the environment each week in many successful programmes around the world (Neuenschwander, 1994; Vargas-Terán et al., 1994; Dyck et al., 2005; Tabashnik et al., 2010; Feldmann et al., 2021; Vargas-Terán et al., 2021) (see Scott et al., Chapter 17, this volume). In theory, the release of self-limiting GM insects should have similar risks, although assessment must be conducted on a case-by-case basis for each insect species and combination of engineered traits. However, the approaches used in the release of beneficial insects and SIT programmes can serve as a useful precedent for countries considering how to regulate GM insects (Mumford, 2012), although to date there has been a preference for regulators to use the legislative approach for GMOs rather than any other precedent.

25.3 Genetically Modified Insects – Current Progress

Since the first GM arthropod release, a predatory mite in the USA in 1996 (Hoy, 2000), and subsequent open field trials of a GM pink bollworm that was also irradiated for sterility in the mid-2000s (Simmons et al., 2011), the past few years have seen rapid progress in the testing of innovative genetic vector control strategies. Table 25.1 shows examples of modified insects and their current status with regard to open field release. Most of these releases were carried out using insects developed by Oxitec Ltd, and which carried self-limiting genes designed to cause mortality in the offspring of the released male insects (see Morrison, Chapter 23, this volume).

These releases have used GM mosquitoes and moths that were regarded as 'self-limiting'

in the environment; however, research is also underway with GM mosquitoes that contain gene drives (see Bottino-Rojas and James, Chapter 11, this volume). Gene drives can be defined (NASEM, 2016) as:

> Thus, the result of a gene drive is the preferential increase of a specific genotype, the genetic makeup of an organism that determines a specific phenotype (trait), from one generation to the next, and potentially throughout the population.

Although there are natural gene drives (Burt and Trivers, 2006), those that use introduced genetic elements fall under the definitions of LMOs and will be subject to regulation. Synthetic gene drives include the use of transposable elements, underdominance, meiotic drive, homing endonuclease genes, and CRISPR/Cas systems, resulting in diverse phenotypes (Alphey et al., 2020) (see Raban and Akbari, Chapter 8; Champer, Chapter 9, this volume). Depending on the genetic system introduced, these could spread and persist in the target population and potentially beyond into adjacent populations (James, 2005; Sinkins and Gould, 2006; Alphey et al., 2013; Alphey, 2014). Since 2012, the pace of research in GM insects has accelerated with the use of CRISPR/Cas gene editing systems (Gantz and Bier, 2015; Hammond et al., 2016; Lester et al., 2020; Simoni et al., 2020) (see Concha and Papa, Chapter 7, this volume).

These are likely to provide additional challenges for regulators to address, due to their potential for persistence and replication in mosquito populations. The pace of the research has also brought about an abundance of publications regarding criteria for the conduct of gene drive research as well as major guidance documents from international organizations and regulatory agencies, which will be discussed later in this chapter.

25.4 Common Features of Regulatory Systems

Irrespective of the national regulatory framework or the enabling legislation on which the regulatory framework is built,

there are common features of most regulatory systems aimed at regulating GMOs.

25.4.1   Information requirements

There are commonalities in the types of information required in regulatory applications, which fall into broad categories (below); however, it is important that the *specific* requirements in the regulatory application are adhered to, as this broad categorization cannot capture all the details needed. Scientific evidence and literature citations, as well as other supporting materials, should be used to verify the narrative information provided.

- **Administrative information**. This covers the applicant or principal investigator details, the organizational structure and location of the research, previous regulatory outcomes (including risk assessments and regulatory opinions) regarding the same or similar organism(s), information regarding review by institutional biosafety committees (IBC) and in some cases financial security of the applicant or the applicant organization.
- **Information related to the parental or recipient insect(s) that are being modified**. This includes background information regarding the geographical range and habitat of the insect, the biology of the insect including modes of reproduction, and ecology of the insect. For an example of the type of information required see the OECD consensus biology document on *Ae. aegypti* (OECD, 2018).
- **Details regarding the genetic modification**. This includes all vectors and genetic components used to generate the GM insect and their donor organisms and function in the GM insect, transformation method and lineage of the GM insect that is the subject of the application.
- **Information on the GM insect and the new traits resulting from the genetic modification**. This includes molecular characterization and phenotypic characterization of the GM insect in comparison with the parental/recipient insect or other suitable comparator, and a summary of results of previous research on the GM insect from other locations or contained-use experiments.
- **Receiving environment**. This includes the geographical locations of either the contained-use facility or the location of the environmental release site, including high-quality maps, details regarding biophysical and hydrogeological characteristics, climate, ecological characteristics, biological diversity and habitats and centres of origin of species.
- **Detailed protocols** relating to the research to be undertaken, including storage, handling, release and transport.
- **Measures to be adopted in the case of an emergency** during the research, such as inadvertent release of the GM insect, or natural disasters such as hurricanes, earthquakes, etc.
- **A risk assessment**. Most regulatory authorities require a risk assessment to support the application. Some authorities will conduct this themselves based on the information in the application; others expect the applicant to provide a risk assessment, depending on the legislative requirements.
- **Risk management measures** based on potential risks identified during the risk assessment.
- **Post-release monitoring methods**. In the case of environmental releases, monitoring methods and frequency of monitoring should be provided, along with information on the sensitivity and robustness of the detection methodologies.

25.4.2   Risk assessment

The use of systematic risk assessment is one such element based on the available scientific and technical data. Scientifically sound risk assessment is widely used in many disciplines where risk needs to be assessed, such as GM crops (Hill and Sendashonga, 2003; Nickson, 2008; Wolt *et al*., 2010), food

safety (Codex Alimentarius Commission, 1995) and chemical safety (Arendt and Lorenzo, 2000; EPA, 2000). The universal steps in risk assessment have been recognized internationally through the Cartagena Protocol on Biosafety Road Map on Risk Assessment, the European Food Safety Authority (EFSA) in its Guidance Document on Environmental Risk Assessment for Genetically Modified Animals (EFSA, 2013), and recent evaluation of risk assessments for gene drive (EFSA, 2020) and the WHO/FNIH Guidance Framework for Testing Genetically Modified Mosquitoes (WHO/TDR and FNIH, 2014; WHO/TDR, 2021). The WHO 2021 guidance document on GM mosquitoes considers that an overall assessment endpoint for a risk assessment of GM mosquitoes should be that the use 'causes no more harm' than current practice. This is similar to the framework for risk assessment and analysis that has been adopted for GM organisms by the Australian Office of the Gene Technology Regulator (OGTR) and serves as a reference point for the assessment of mosquitoes, as their wild counterparts already cause harm to human and animal health through the transmission of diseases. Commonality in risk assessment approaches between authorities in Australia, Europe and the United Nations Environment Program (UNEP) (the Cartagena Protocol on Biosafety) for GM insects was also reviewed in Turner *et al*. (2018).

The core principles of risk assessment are maintained across these various global guidance documents (problem formulation, hazard and exposure characterization and risk characterization and management). The use of problem formulation as the key initial step identifies the important questions for risk characterization by stating the assumptions underlying the risk assessment in a structured, transparent and systematic way. Several problem formulation exercises have been carried out for gene drive mosquitoes in recent years. Roberts *et al*. (2017) reported on a problem formulation workshop held in the USA in 2016, on gene drive in *Anopheles* mosquitoes. Teem *et al*. (2019) reported the results of four African-based regional consultations on gene drive

mosquitoes for the reduction of malaria transmission, which identified human health and biodiversity as relevant protection goals, with common themes of potential harms that included the potential to increase malaria or other mosquito-borne diseases and the reduction of mosquito predators or impacts on other mosquitoes, including as potential harms to biodiversity. These and other thematic areas, such as animal health and water quality, were further investigated by Connolly *et al*. (2021), using a specific example of a CRISPR/Cas9 population suppression gene drive in a simulated field release in West Africa, including preparation of 46 cause–effect pathways, with risk hypotheses and an analysis plan for subsequent experimental investigation.

The six common steps in risk assessment are shown diagrammatically in Fig. 25.1.

The risk assessment should be carried out on a case-by-case basis, where the required information will vary depending on the type of insect concerned, the introduced trait(s), the intended use and the environment in which it will be used. It should be based on a comparison with the unmodified insect, or conventional controls or other alternatives, if no suitable insect comparator exists. The risk assessor should seek to determine what effect the genetic modification has on the insect and whether this makes it more likely to cause harm to humans, animals or the environment than the unmodified insect or conventional pest control methods. Mumford (2012) indicated that release of GM insects into the environment poses two broad risk issues: environmental risks associated with the introduction of large numbers of mass-reared insects (for programmes based on inundative releases) and specific risks that may be associated with the process of genetic modification. For the former there appear to be many precedents regarding their safe use from pre-existing biological control programmes that provide templates for the evaluation of GM insects. The risk assessment then serves as one of the inputs for decision making for regulators where the recommendations derived from the risk assessment are taken
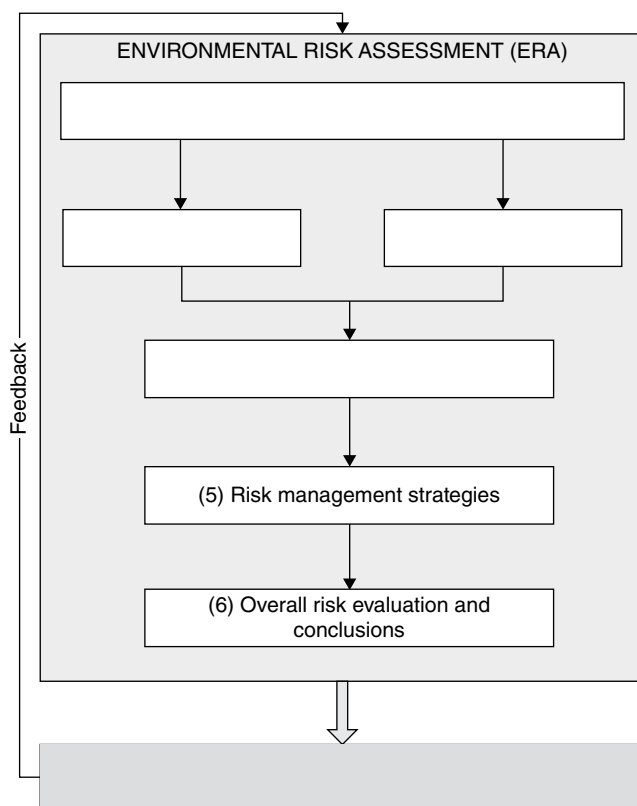
Fig. 25.1. The six common steps in risk assessment. From EFSA (2013), CC BY-ND 4.0.

The figure contains the following labelled boxes:

- ENVIRONMENTAL RISK ASSESSMENT (ERA)
- (5) Risk management strategies
- (6) Overall risk evaluation and conclusions
- Feedback

into account in accordance with the country's policies and environmental or human protection goals. Risk management measures with other issues such as socio-economic impact, public awareness and liability considerations may also be taken into account during the decision-making process. There is a chapter dedicated to risk analysis in this volume (see Hayes and Quinlan, Chapter 28) and consequently this will not be discussed further here, except to say that it is an integral and important part of the regulatory process, providing significant evidence for decision makers.

25.5 Guidance Documents on Gene Drives

In recent years many recommendations and guidance documents from organizations and regulatory authorities have emerged on the use of gene drive organisms, predominantly focused on mosquitoes for vector control applications. It is important to mention that the majority of current gene drives would fit the statutory definitions of genetically modified organisms and therefore fall under existing regulations of GMOs. The WHO and CPB guidance (mentioned above in section) as well as the proposed harmonization documents from the WA-IVM initiative, are key guidance documents in this regard. Reports from NASEM (2016) and EFSA (2020) are also regularly cited.

The US National Academies of Sciences, Engineering, and Medicine (NASEM) published a report on gene drives (NASEM, 2016) which recommended robust risk assessment including the use of quantitative ecological risk assessment tools to estimate the probability of direct, indirect cumulative

and long-term potential harms and benefits, via cause-and-effect pathways, in comparison with alternative strategies.

EFSA has also recently evaluated whether its previously published guidance on risk assessment (EFSA, 2013) is adequate for gene drive organisms, concluding that the existing guidance is appropriate for risk assessment of the release of gene drive insects, but more specificity is required to address particular areas unique to gene drives, such as molecular characterization, the use of modelling to predict scenarios in advance of release, and an assessment of suitable information to gather in post-market monitoring, due to the spatial and temporal scope of gene drive spread and establishment in the environment (EFSA, 2020). Devos *et al.* (2020) also discussed development of practical risk assessment guidance for gene drive organisms.

Since the first edition of this chapter, national governments and authorities have issued various statements and reports on gene drives or have revised existing laws to include gene drives and these are listed in Table 25.2. In addition to the national regulatory standards in this table, self-governance literature by scientists for the use of gene drives in GM insects has been extensive, across aspects of governance, risk assessment, contained use, field release, ethics and public engagement. Several researchers have published on self-governance of gene drives (Carter and Friedman, 2016; Adelman *et al.*, 2017; Emerson *et al.*, 2017; Rudenko *et al.*, 2018) as well as guidance for field trials, safety and efficacy testing and site selection (Benedict *et al.*, 2008; James *et al.*, 2020; Kormos *et al.*, 2020; Long *et al.*, 2020). Common features include adherence to regulatory requirements, preparation of safety and efficacy data, risk–benefit assessment, monitoring and risk management planning, transparency and openness and stakeholder engagement. Ethics and public engagement, which help to shape the values often involved in risk management, are important considerations in gene drive research, but will not be further addressed in this chapter.

With regard to aspects of contained use, Benedict *et al.* (2018) gave specific consideration to the measures that exist in the American Committee of Medical Entomology/American Society of Tropical Medicine and Hygiene *Arthropod Containment Guidelines* (ACME/ASTMH, 2019) that are appropriate for gene drive arthropods, along with a review of existing standards for arthropod containment from Australia/New Zealand, UK, USA and Canada. There was a strong convergence across these standards in terms of recommendations and nomenclature, leading to the conclusion that the approach for the containment of arthropods is common and effective, and is based around waste control for all arthropod life stages, control of movement of equipment and staff, and control of physical structures such as doors, windows, ventilation and drainage, as well as a case-by-case risk assessment for potential for establishment in the case of inadvertent release. However, for gene drive-containing arthropods, consideration should also be given to strain and insectary design and facility management, including the use of unique visual fluorescent marker genes, regular strain authentication, housing insects to prevent probability of cross-mating, and routine management checks. The routine use of IBCs to examine proposals for contained-use research with gene drive-containing arthropods would also be necessary. This later requirement is legally necessary in the EU and has also recently been considered for research with GM insects in the USA (O'Brochta *et al.*, 2020).

25.6 Emerging Themes in Regulation of GM Insects

Broad themes emerge for the regulation of GM insects, in addition to those mentioned above regarding gene drives.

Firstly, the release of GM insects for control of pest populations is no longer novel. Over a billion GM insects have been released, mostly GM *Ae. aegypti* in Brazil. Brazil has taken the lead in regulating GM insects, with three different GM insect strains having achieved commercial biosafety approval (two *Ae. aegypti* strains and one

Table 25.2. National government and authority decisions and reports on gene drive organisms.

Date	Country	Document title	Website link (all accessed 4 April 2022)
2015	UK	Science and Technology Committee of the UK Parliament Report on Genetically Modified Insects	https://publications.parliament.uk/pa/ld201516/ldselect/ldsctech/68/6802.htm
2017	France	Scientific Opinion of the French High Council for Biotechnology on the use of Genetically Modified Mosquitoes for Vector Control	http://www.hautconseildesbiotechnologies.fr/en/avis/avis-relatif-a-lutilisation-moustiques-gm-dans-cadre-lutte-antivectorielle
2017	New Zealand	Report of the Royal Society Te Aparangi Gene Editing panel on Use of Gene Editing to create Gene Drives for Pest Control in New Zealand	https://royalsociety.org.nz/assets/Uploads/Gene-editing-in-pest-control-technical-paper.pdf
2017	Australia	Synthetic gene drives in Australia; Implications of Emerging Technologies	https://www.science.org.au/support/analysis/reports/synthetic-gene-drives-australia-implications-emerging-technologies
2019	Australia	Office of the Gene Technology Regulator; guidance for IBCs: Regulatory requirements for contained research with GMOs containing engineered gene drives	http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/53139D205A98A3B3CA257D4F00811F97/$File/Guidance%20on%20gene%20drives.pdf
2017	Norway	Statement of the Norwegian Biotechnology Advisory Board on Gene Drives	https://www.bioteknologiradet.no/english/
2018	Netherlands	Netherlands Commission on Genetic Modification Report on Experiences with Gene Drive Systems that may inform an Environmental Risk Assessment	https://cogem.net/app/uploads/2019/07/CGM-2018-03-Report-Gene-Drives-met-kaft1.pdf
2018	Netherlands	National Institute for Public Health and the Environment (RIVM): Risk assessment Method for activities involving organisms with a gene drive under Contained Use (Report 2018-0090)	https://www.rivm.nl/bibliotheek/rapporten/2018-0090.pdf
2018	Brazil	National Biosafety technical Commission Normative Resolution No.16 of January 15, 2018 – update to Law 11.105 to include gene drive	http://bch.cbd.int/database/record.shtml?documentid=113509
2020	Austria	Environment Agency Report on Gene Drive Organisms: Implications for the Environment and Nature Conservation	https://www.umweltbundesamt.at/fileadmin/site/publikationen/rep0705.pdf
2020	Switzerland	Swiss Academies of Science: Fact Sheet on Gene Drives; Benefits, Risks and Possible Applications	https://scnat.ch/en/uuid/i/045a3073-e301-5215-a0a0-3ca3d5b85a78-Gene_drives%3A_benefits%2C_risks%2C_and_possible_applications
2021	Germany	Genetic Engineering Safety Ordinance (GenTSV) – update to include gene drive organisms in contained use	https://gsetz.de/norm/gentsv%202021.html [in German]

S. frugiperda strain). Several other countries have granted permits for field trials, most notably the USA, the Cayman Islands, Burkina Faso, Malaysia and Panama.

Secondly, the importance of the positions of developing countries in shaping the global regulatory frameworks around GM insects has become apparent, particularly in Africa, where the proactive development of regulatory guidance for gene drive mosquitoes is supported at the highest political levels (Glover *et al.*, 2018).

Thirdly, the development of quantitative risk assessment tools (particularly for gene drive-containing organisms) is a growing theme in the regulation of GM insects, following recommendations from several major opinion shapers (NASEM, 2016; Hayes *et al.*, 2018; James *et al.*, 2018; EFSA, 2020).

Finally, the incorporation of socio-economic considerations and public participation in risk assessment, decision making and community engagement (Hartley *et al.*, 2019) is a key theme to emerge in the past decade. These elements may be particularly relevant to applications that are intended to provide public health benefits.

However, these also represent some significant challenges for academic groups, start-up companies and regulators. The use of quantitative risk assessment tools, while well established in some regulatory systems such as the US EPA for chemical assessment (EPA, 2017), remains nascent or non-existent for authorities that have based their regulatory systems on the Cartagena Protocol for Biosafety. How they will provide resources, build capacity, navigate and validate these tools remains to be seen, although as discussed previously the concept of quantitative assessment has some merit for gene drive-containing organisms for probability assessment and sensitivity analysis.

The incorporation of socio-economic considerations in the regulation of GMOs has been debated for some time both in international fora, as it is included in Article 26.1 of the Cartagena Protocol on Biosafety, and in the scientific literature (Binimelis and Myhr, 2016; Racovita, 2017; Chaturvedi and Srinivas, 2019), but details on how the assessment is to be carried out remain scant.

The engagement of stakeholders is generally accepted as a necessary part of the use of GM insects in agriculture and vector control. However, detailed guidance on how public engagement should be carried out is not well defined. Schairer *et al.* (2019) reviewed methodologies in 14 examples of stakeholder and community engagement projects in emerging vector control technologies and found no clear pattern regarding timing of engagement activities, but proposed three types of engagement: (i) engagement to inquire (learning from a target group); (ii) engagement to influence (efforts to educate or deliver messages with the intention of influencing decisions or changing behaviours); and (iii) engagement to involve (decision-making process that is open to the target group), each with differing methods, evaluation and reporting. Thizy *et al.* (2019) published recommendations from a multidisciplinary team on guidance for stakeholder engagement in gene drive projects, attempting to address recommendations from the NASEM 2016 report *Gene Drives on the Horizon: Advancing Science, Navigating Uncertainty and Aligning Research with Public Values*.

Burgess *et al.* (2018) reviewed engagement largely through a legislative and regulatory lens, concluding that successful engagement will have demonstrable outcomes and decisions and that consensus opinions should not be expected, but that the legitimacy of the engagement, enhancing openness and inclusiveness in the process, was important. However, there is no universal approach and government agencies frequently do not have a sufficiently wide mandate to engage in resource-intensive public engagement, although their guidance documents should commit to some level of public engagement.

25.7 Regulatory Gaps and Overlaps

The use of GM insects can offer potential advantages over conventional pest control strategies. In particular, they are able to exploit the natural behaviour of the insect to reach breeding sites that cannot be reached

by chemical applications, are expected to have fewer off-target effects than broad-spectrum pesticide use and may help to prevent reintroduction of the pest insect on a long-term basis (as has been previously proven by SIT with irradiated insects).

As expected for new technologies where regulation often lags behind social expectations and science (NRC, 1989; Lehane and Aksoy, 2012; Oye *et al*., 2014), the field use of GM insects has highlighted several issues including regulatory gaps and overlaps, as follows.

1. Regulatory uncertainty, or lack of experience, can provide a vacuum that can be (and is) exploited by groups opposed to new technologies, especially genetic engineering (ideologically or otherwise), through but not limited to both legal and communication channels (such as social media, internet campaigns, petitions, etc.). The regulatory process is often where this heat is brought to bear, because it is a key gatekeeper to deployment of new technologies.

2. It is not always clear which legislative framework applies to some species of GM insects (e.g., mosquitoes); often, submitting an application for a field release is the only way to determine the correct regulatory pathway, as agencies have a mandate to act on the information before them. This could result in either agency oversight, or a determination that the agency is not the correct agency/legislative framework under which to operate. This can often add considerable uncertainty and delay to the process. An example of this occurred when USDA Veterinary Services issued a statement of No Jurisdiction for an application for open field release in the USA of *Ae. aegypti* mosquitoes engineered with conditional lethal and fluorescent marker traits. The jurisdiction was subsequently taken up by the US FDA Center for Veterinary Medicine (FDA-CVM) in accordance with their Guidance for Industry (GFI) Number 187 on GM animals (FDA-CVM, 2015). After five years of oversight by FDA-CVM (and the granting of a field release permit) it was agreed that GM insects would henceforth be regulated based on their intended use, with those that were to

be marketed as pest control or pesticidal agents to be regulated by the US EPA (FDA-CVM, 2017). At the time of writing (in 2021), a second *Ae. aegypti* mosquito strain is being regulated by US EPA and has begun its first field trials in Florida. However, it is not just GM mosquitoes that fall between regulatory boundaries. The case of the GM zebrafish in the USA (Box 25.1) and the use of the intracellular bacterium *Wolbachia pipientis* in *Ae. aegypti* in Australia (described below) are two such examples. The use of the intracellular bacterium *W. pipientis*, which is found in many insect species, to induce refractoriness of the *Ae. aegypti* mosquito to the dengue virus (Walker *et al*., 2011) also fell between regulatory boundaries for field release in Australia (Hoffman *et al*., 2011). As the mosquito was artificially infected with a bacterium that was naturally occurring, albeit not in that species, it did not fall under the genetic modification legislation, but was eventually regulated as a veterinary chemical product (Murphy *et al*., 2010; De Barro *et al*., 2011).

3. Legal frameworks and requirements invoked may differ based on the jurisdictional level (e.g., federal/national, state/provincial and local levels), which has the potential effect of further delaying decision making, even when a positive scientific decision has been made by a national regulator. This may ultimately prevent a GM insect product being sold.

4. Navigating the shipping requirements for GM insects is often an issue that is frequently more complex than anticipated. Requirements for the shipment and transport of live insects is inconsistent, although some sectorial guidance is available, for example for honey bees for pollination purposes (guidance provided by the World Organization for Animal Health, OIE), or for biocontrol agents (guidance provided by the International Standard for Phytosanitary measures, ISPM3). Transport of GMOs (including insects) is subject to the overarching Cartagena Protocol on Biosafety, with labelling and packing requirements for safe handling (see Periera, Chapter 27, this volume). Finding a courier service that is capable and willing to carry live GM insects and ship

them in an expedited manner adds further complexity.

5. Different agencies regulate imports into countries, often under quarantine for some insect pest species, and several different permits may be required for importing live insects. These may have conflicting requirements, such as packaging, transport routes, labelling, health certificates, etc.

6. GM insects are being developed largely in the public sector, funded by academic grants or philanthropic organizations, or by small companies with limited resources, and products are aimed at serving public health or food security needs in developing countries. A negative effect of compliance with complex, costly and time-intensive regulation is the virtual exclusion of public-sector researchers from the development of these products.

7. As GM insects are already pest species and are controlled in the environment with pesticides and other methods that are not environmentally neutral, regulatory agencies should consider including risk–benefit analysis against current pest control alternatives for these insects in their decision making, an example of this being the US Environmental Impact Statements for GM pink bollworm and fruit flies, and requirements from several African countries to conduct broader environmental impact evaluations that assess both risks and benefits for the biophysical, health and socio-economic environments.

8. Developing mutual recognition of data by different countries or regional regulatory approaches, such as the WA-IVM guidance documents, would serve to foster a harmonized framework for the assessment of GM insects, comparable across countries, as recommended by WHO. For the use of gene drive technologies in insects, this is especially

---

Box 25.1. Case study of the genetically modified zebrafish in the USA.

When the zebrafish, *Danio rerio*, was genetically transformed to express fluorescent proteins, a company in Texas asked US regulatory officials for permission to sell them as a novelty in pet stores in the USA under the trade name GloFish®. The regulatory officials in all US agencies under the Co-ordinated Framework for the Regulation of Biotechnology, USDA, EPA and FDA examined the case and found it was not specifically mentioned in the statutes; therefore, they declined to regulate it. Zebrafish is a tropical fish that cannot survive at the latitudes found in the USA, thus it is not a pest, not a crop, not a food and not an invasive species.

The statement on the US Food and Drug Administration website is as follows.

Because tropical aquarium fish are not used for food purposes, they pose no threat to the food supply. There is no evidence that these genetically engineered zebra danio fish pose any more threat to the environment than their unmodified counterparts which have long been widely sold in the United States. In the absence of a clear risk to the public health, the FDA finds no reason to regulate these particular fish.

The world media had a field day writing stories about this case, such as 'the one that got away' (Anonymous, 2004). However, State regulation was also involved and in California the Fish and Game Commission was asked to approve of sales of GloFish. The company selling GloFish describes the Californian decision on its website:

GloFish® in California: You may be wondering why GloFish® fluorescent fish are allowed everywhere in the United States except California. As many people know, our fish received a positive recommendation from the California Department of Fish and Game in November 2003, and the California Fish & Game Commission voted to move forward with the process of exempting our fish from their ban on biotech aquatic organisms in April 2004. However, we were subsequently advised by Commission attorneys that state law in California would require the completion of a formal ecological review to comply with the California Environmental Quality Act before the Commission could move forward with the approval process. Unfortunately, this review would be extremely expensive, involve procedural uncertainty, and likely take several years to complete. Due to the excessive cost and time involved in that process, as well as the uncertainty, we have decided not to engage the review. We regret this situation, but unfortunately cannot afford, at this time, to make the enormous investment necessary to perhaps be able to market our fish in California.

important given the potential for transboundary movements by spontaneous flight across political boundaries.

25.8   Conclusions

The use of GM insects represents a novel and innovative tool to address insect-borne diseases of humans and crop pest losses and some applications (*Ae. aegypti* and *S. frugiperda*) have already received commercial biosafety approvals. The purpose of regulating GM insects, including those with engineered gene drives, remains the same: to provide an assurance of safety of a product prior to entry into general and widespread use, in compliance with the applicable national, local and other relevant laws. These laws and regulations derive from the values of society at large and thereby define common protection goals at the highest level. Thus, regulations determine the scope of the determinations of safety being made on a case-by-case basis and are sovereign to each country. Determinations should be based on structured, systematic evidence-based evaluations using well-known approaches such as risk analysis using both quantitative and qualitative tools. The use of modelling of scenarios will become increasingly important for the evaluation of gene drives. Although there are common international governance frameworks such as the Cartagena Protocol on Biosafety or guidance documents on GM insects such as those provided by WHO or EFSA, some less developed countries that may have the most to gain from the potential use of these technologies still have to develop or adapt their own national policies and regulatory requirements as well as build capacity to implement their national frameworks. Proportionality, predictability and transparency in regulation are essential, especially in relation to informational requirements, timescales for review and outcomes. This will help build confidence in the regulatory system, for both the regulated community and societies, although it is unlikely to convince those who are opposed to the introduction of such technologies. Going further, seeking harmonization of regulatory requirements, transparency and portability of data and sharing of regulatory opinions will further advance regulatory capacity in the evaluation of GM insects and will be increasingly important for gene drive technologies that may transcend political boundaries.

References

ACME/ASTMH (American Committee of Medical Entomology/American Society of Tropical Medicine and Hygiene) (2019) Arthropod Containment Guidelines, Version 3.2. *Vector Borne Zoonotic Diseases* 19(3), 152–173. doi: 10.1089/vbz.2018.2431

Adelman, Z., Akbari, O., Bauer, J., Bier, E., Bloss, C. *et al.* (2017) Rules of the road for insect gene drive research and testing. *Nature Biotechnology* 35, 716–718. doi: 10.1038/nbt.3926.

AHTEG (2020) *Report of the Ad Hoc Technical Expert Group on Risk Assessment*. Item 7 of provisional agenda for meeting 30 March–3 April 2020. Convention on Biological Diversity, Montreal, Canada. Available at: https://www.cbd.int/cp-ra-ahteg-2020-01-05-en (accessed 23 April 2022)

Alphey, L. (2014) Genetic control of mosquitoes. *Annual Review of Entomology* 59, 205–224. doi: 10.1146/annurev-ento-011613-162002.

Alphey, L., McKemey, A., Nimmo, D., Neira Oviedo, M., Lacroix, R. *et al.* (2013) Genetic control of *Aedes* mosquitoes. *Pathogens and Global Health* 107, 170–179. doi: 10.1179/2047773213Y.0000000095.

Alphey, L.S., Crisanti, A., Randazzo, F. and Akbari, O.S. (2020) Opinion: Standardizing the definition of gene drive. *Proceedings of the National Academy of Sciences USA* 117, 30864–30867. doi: 10.1073/pnas.2020417117.

Anonymous (2004) The one that got away. Nature Biotechnology 22, 1. https://doi.org/10.1038/nbt0104-1

Arendt, J.S. and Lorenzo, D.K. (2000) *Evaluating Process Safety in the Chemical Industry: A Users Guide to Quantitative Risk Assessment*. CPPC Concept Books. Wiley-AIChE, Hoboken, New Jersey.

Asadi, R., Elaini, R., Lacroix, R., Ant, T., Collado, A. *et al.* (2019) Preventative releases of self-limiting *Ceratitis capitata* provide pest suppression and protect fruit quality in outdoor netted cages. *International Journal of Pest Management* 66(2), 1–12. doi: 10.1080/09670874.2019.1601293.

AU (2017) *Decision on AIDS Watch Africa (AWA) Report*. Decision No. Assembly/AU/Dec.649(XXIX). Assembly of the African Union Twenty-Ninth Ordinary Session, 3–4 July 2017, Addis Ababa, Ethiopia

AU (2018) *Executive Council Decision on Specialized Technical Committees on Education, Science and Technology*. Decision No. EX.CL/Dec. 987(XXXII). Executive Council Thirty-Second Ordinary Session, 25–26 January 2018. African Union, Addis Ababa, Ethiopia

AUDA-NEPAD (2018) *Gene Drives for Malaria Control and Elimination in Africa*. Report of the High-Level African Union Panel on Emerging Technologies (APET). African Union Development Agency – New Partnership for Africa's Development (NEPAD), Midrand, Gauteng, South Africa.

AUDA-NEPAD (2020) *Inauguration and first meeting of WA-IVM Technical Working Groups*. AUDA-NEPAD and WAHO, April 2019, Abidjan, Côte d'Ivoire. Available at: https://www.nepad.org/news/inauguration-and-first-meeting-of-wa-ivm-technical-working-groups (accessed 13 July, 2021)

Barreto, M.L., Teixeira, M.G., Bastos, F.I., Ximenes, R.A., Barata, R.B. and Rodrigues, L.C. (2011) Successes and failures in the control of infectious diseases in Brazil: social and environmental context, policies, interventions, and research needs. *The Lancet* 377, 1877–1889. doi: 10.1016/S0140-6736(11)60202-X

Beech, C.J., Mumford, J.D., Quinlan, M.M. and Capurro, M.L. (2011) Update: Deployment of Innovative Genetic Vector Control Strategies including an update on the MosqGuide Project. *Asia-Pacific Journal of Molecular Biology & Biotechnology* 19, 101–106.

Benedict, M., D'Abbs, P., Dobson, S., Gottlieb, M., Harrington, L. *et al.* (2008) Guidance for contained field trials of vector mosquitoes engineered to contain a gene drive system: recommendations of a Scientific Working Group. *Vector-Borne and Zoonotic Diseases* 8, 127–164. doi: 10.1089/vbz.2007.0273

Benedict, M.Q., Burt, A., Capurro, M.L., De Barro, P., Handler, A.M. *et al.* (2018) Recommendations for laboratory containment and management of gene drive systems in arthropods. *Vector-Borne and Zoonotic Diseases* 18, 2–13. doi: 10.1089/vbz.2017.2121

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W. *et al.* (2013) The global distribution and burden of dengue. *Nature* 496, 504–507. doi: 10.1038/nature12060

Bhattacharya, S.K. (2002) Precautionary principle under biosafety protocol: needs for a cautious approach. *Economic and Political Weekly* 37, N, 2441–2446.

Binimelis, R. and Myhr, A.I. (2016) Inclusion and implementation of socio-economic considerations in GMO Regulations: needs and recommendations. *Sustainability* 8, 62. doi: 10.3390/SU8010062

Botha, A.S., Erasmus, A., du Plessis, H. and Van den Berg, J. (2019) Efficacy of Bt maize for control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in South Africa. *Journal of Economic Entomology* 112, 1260–1266. doi: 10.1093/jee/toz048

Bradshaw, C.J.A., Leroy, B., Bellard, C., Roiz, D., Albert, C. *et al.* (2016) Massive yet grossly underestimated global costs of invasive insects. *Nature Communications* 7. doi: 10.1038/ncomms12986.

Burgess, M.M., Mumford, J.D. and Lavery, J.V. (2018) Public engagement pathways for emerging GM insect technologies. *BMC Proceedings* 12, 29–36. doi: 10.1186/s12919-018-0109-x

Burt, A. and Crisanti, A. (2018) Gene drive: evolved and synthetic. *ACS Chemical Biology* 13, 343–346. doi: 10.1021/acschembio.7b01031

Burt, A. and Trivers, R. (2006) *Genes in Conflict: The Biology of Selfish Genetic Elements*. The Belknap Press of Harvard University Press, Cambridge, Massachusetts

Cantley, M. (2012) European attitudes on the regulation of modern biotechnology and their consequences. *GM Crops and Food* 3(1), 40–47. doi: 10.4161/gmcr.19264

Carter, S.R. and Friedman, R.M. (2016) *Policy and Regulatory Issues for Gene Drives in Insects*. J. Craig Venter Institute, La Jolla, California, and Rockville, Maryland.

Carvalho, D.O., McKemey, A.R., Garziera, L., Lacroix, R., Donnelly, C.A. *et al.* (2015) Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. *PLoS Neglected Tropical Diseases* 9, e0003864. doi: 10:1371/journal.pntd.0003864

CAST (2013) *Impact of the Precautionary Principle on Feeding Current and Future Generations*. CAST, Ames, Iowa. Available at: https://www.cast-science.org/new-cast-publication-examines-the-impact-of-the-precautionary-principle/ (accessed July 12, 2021).

CBD (2000) *Cartagena Protocol on Biosafety to the Convention on Biological Diversity*. *Text and Annexes*. Secretariat of the Convention on Biological Diversity, Montreal, Canada.

CBD (2018a) *Parties to the Cartagena Protocol and its Supplementary Protocol on Liability and Redress*. Convention on Biological Diversity, Montreal, Canada. Available at: *https://bch.cbd.int/protocol/parties/* (accessed July 13, 2021)

CBD (2018b) *Decision adopted by the Parties to the Cartagena Protocol on Biosafety. 9/13. Risk assessment and risk management (Articles 15 and 16)*. Convention on Biological Diversity, Montreal, Canada. Available at: cbd.int/doc/decisions/cp-mop-09/cp-mop-o9-dec-13-e.pdf (accessed 25 April 2022)

CDC (2021) *CDC Import Permit Program. Centers for Disease Control and Prevention*, US Department of Health & Human Services, Washington DC. Available at: https://www.cdc.gov/cpr/ipp/about.htm (accessed July 13, 2021)

Chandramohan, D., Zongo, I., Sagara, I., Cairns, M., Yerbanga, R.-S. *et al.* (2021) Seasonal malaria vaccination with or without seasonal malaria chemoprevention. *New England Journal of Medicine* 385, 1005–1017. doi: 10.1056/nejmoa2026330

Chaturvedi, S. and Srinivas, K.R. (2019) *Socio-Economic Impact Assessment of Genetically Modified Crops*. *Springer Singapore.* doi: 10.1007/978-981-32-9511-7

Codex Alimentarius Commission (1995) Guidelines for the Application of the Hazard Analysis Critical Control Point (HACCP) System. In: *Codex Alimentarius Vol. 1B: General Requirements (food hygiene)*. FAO/WHO, Rome, pp. 21–30.

COMESA (2019) *COMESA/ACTESA to revive regional policy on biotechnology and biosafety*. Common Market for Eastern and Southern Africa. Available at: https://www.comesa.int/comesa-actesa-to-revive-regional-policy-on-biotechnology-and-biosafety/ (accessed October 14, 2021)

Concha, C., Palavesam, A., Guerrero, F.D., Sagel, A., Li, F. *et al.* (2016) A transgenic male-only strain of the New World screwworm for an improved control program using the sterile insect technique. *BMC Biology* 14, 72. doi: 10.1186/s12915-016-0296-8

Connolly, J.B., Mumford, J.D., Fuchs, S., Turner, G., Beech, C., North, A.R. and Burt, A. (2021) Systematic identification of plausible pathways to potential harm via problem formulation for investigational releases of a population suppression gene drive to control the human malaria vector *Anopheles gambiae* in West Africa. *Malaria Journal* 20, 170. doi: 10.1186/s12936-021-03674-6

Cunha, M.S., Costa, P.A.G., Correa, I.A., de Souza, M.R.M., Calil, P.T. *et al.* (2020) Chikungunya virus: an emergent arbovirus to the South American continent and a continuous threat to the world. *Frontiers in Microbiology* 11, 1297. doi: 10.3389/fmicb.2020.01297

Datoo, M.S., Natama, M.H., Somé, A., Traoré, O., Rouamba, T. *et al.* (2021) Efficacy of a low-dose candidate malaria vaccine, R21 in adjuvant Matrix-M, with seasonal administration to children in Burkina Faso: a randomised controlled trial. *The Lancet* 397, 1809–1818. doi: 10.1016/S0140-6736(21)00943-0

De Barro, P., Murphy, B., Jansen, C. and Murray, J. (2011) The proposed release of the yellow fever mosquito, *Aedes aegypti*, containing a naturally occuring strain of *Wolbachia pipientis*, a question of regulatory responsibility. *Journal für Verbraucherschutz und Lebensmittelsicherheit* 6 (Suppl. 1), S33–S40.

Devos, Y., Bonsall, M.B., Firbank, L.G., Mumford, J., Nogué, F. and Wimmer, E.A. (2020) Gene drive-modified organisms: developing practical risk assessment guidance. *Trends in Biotechnology* 39(9), 853–856. doi: 10.1016/j.tibtech.2020.11.015.

Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) (2005) *Sterile Insect Technique: Principles and practice in Area-Wide Integrated Pest Management*. Springer, Dordrecht, Netherlands.

EC (2000) *First Report on the Harmonisation of Risk Assessment Procedures: Part 1: The Report of the Scientific Steering Committee's Working Group on Harmonisation of Risk Assessment Procedures in the Scientific Committees advising the European Commission in the area of human and environmental health*. European Commission Scientific Steering Committee, Brussels.

Ecuru, J. (2017) A pathway for biosafety regulation of GMOs in sub-Saharan Africa. In: *Genetically Modified Organisms in Developing Countries: Risk Analysis and Governance*. Cambridge University Press, 283–293. doi: 10.1017/9781316585269.025.

EFSA (2013) European Food Safety Authority Panel on Genetically Modified Organisms Guidance on the environmental risk assessment of genetically modified animals. *EFSA Journal* 11(5), 3200. doi: 10.2903/j.efsa.2013.3200.

EFSA (2020) Adequacy and sufficiency evaluation of existing EFSA guidelines for the molecular characterisation, environmental risk assessment and post-market environmental monitoring of genetically modified insects containing engineered gene drives. *EFSA Journal* 18(11), 6297. doi: 10.2903/j.efsa.2020.6297

Emerson, C., James, S., Littler, K. and Randazzo, F. (Fil) (2017) Principles for gene drive research. *Science* 358, 1135LP–1136. doi: 10.1126/science.aap9026.

EPA (2000) *Risk Characterization.* Science Policy Council HANDBOOK. Science Policy Council (SPC) for United States Environmental Protection Agency, Washington, DC.

EPA (2017) Exposure Assessment Tools by Tiers and Types - Deterministic and Probabilistic Assessments. United States Environmental Protection Agency, Washington, DC. Available at: https://www.epa.gov/expobox/exposure-assessment-tools-tiers-and-types-deterministic-and-probabilistic-assessments (accessed July 13, 2021).

FDA-CVM (2015) *CVM GFI #187 Regulation of Genetically Engineered Animals Containing Heritable Recombinant DNA Constructs*. US Food and Drug Administration, Center for Veterinary Medicine, Rockville, Maryland. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents (accessed 25 April 2022)

FDA-CVM (2017) *CVM GFI #236 Clarification of FDA and EPA Jurisdiction Over Mosquito-Related Products. US Food and Drug Administration*, Center for Veterinary Medicine, Rockville, Maryland. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cvm-gfi-236-clarification-fda-and-epa-jurisdiction-over-mosquito-related-products (accessed 13 July 2021).

Feldmann, U., Dyck, V.A., Mattioli, R.C., Jannin, J. and Vreysen, M.J.B. (2021) Impact of tsetse fly eradication programmes using the sterile insect technique. In: Dyck, V.A., Hendrichs, J. and Robinson, A.S. (eds) *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management*. CRC Press, Boca Raton, Florida, pp. 1051–1080.

Gantz, V.M. and Bier, E. (2015) The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. *Science* 348, 442–444. doi: 10.1126/science.aaa5945

Giesen, C., Roche, J., Redondo-Bravo, L., Ruiz-Huerta, C., Gomez-Barroso, D. *et al.* (2020) The impact of climate change on mosquito-borne diseases in Africa. *Pathogens and Global Health* 114, 287–301. doi: 10.1080/20477724.2020.1783865

Glover, B., Akinbo, O., Savadogo, M., Timpo, S., Lemgo, G. *et al.* (2018) Strengthening regulatory capacity for gene drives in Africa: leveraging NEPAD's experience in establishing regulatory systems for medicines and GM crops in Africa. *BMC Proceedings* 12 (Suppl. 8), 19–28. doi: 10.1186/S12919-018-0108-Y

Gorman, K., Young, J., Pineda, L., Márquez, R., Sosa, N. *et al.* (2016) Short-term suppression of *Aedes aegypti* using genetic control does not facilitate *Aedes albopictus*. *Pest Management Science* 72, 618–628. doi: 10.1002/ps.4151

Guidotti, T.L. (2012) Applying the precautionary principle. *Archives of Environmental and Occupational Health* 67(2).

Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C. *et al.* (2016) A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nature Biotechnology* 34, 78–83. doi: 10.1038/nbt.3439

Harris, A.F., McKemey, A.R., Nimmo, D., Curtis, Z., Black, I. *et al.* (2012) Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nature Biotechnology* 30, 828–830. doi: 10.1038/nbt.2350

Hartley, S., Thizy, D., Ledingham, K., Coulibaly, M., Diabaté, A. *et al.* (2019) Knowledge engagement in gene drive research for malaria control. *PLOS Neglected Tropical Diseases* 13, e0007233. doi: 10.1371/JOURNAL.PNTD.0007233.

Hayes, K.R., Hosack, G.R., Ickowicz, A., Foster, S., Peel, D. *et al.* (2018) *Risk Assessment for Controlling Mosquito Vectors with Engineered Nucleases: Controlled field release for Sterile Male Construct*. CSIRO Health and Biosecurity, Hobart, Australia. doi: 10.25919/5c017aa7231b2.

Hill, R.A. and Sendashonga, C. (2003) General principles of risk assessment of living modified organisms: lessons from chemical risk assessment. *Environmental Biosafety Research* 2, 81–88.

Hoffman, A.A., Montgomery, B., Popovici, J., Irurbe-Ormaetxe, I., Johnson, P.H. *et al.* (2011) Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature* 476, 454–456. doi: 10.1038/nature10356

Hoy, M.A. (2000) Transgenic arthropods for pest management programs: risks and realities. *Experimental & Applied Acarology* 24, 463–495. doi: 10.1023/A:1006401225083.

James, A.A. (2005) Gene drive systems in mosquitoes: Rules of the road. *Trends in Parasitology* 21, 64–67. doi: 10.1016/j.pt.2004.11.004

James, S., Collins, F.H., Welkhoff, P.A., Emerson, C., J Godfray, H.C. *et al.* (2018) Pathway to deployment of gene drive mosquitoes as a potential biocontrol tool for elimination of malaria in sub-Saharan Africa: Recommendations of a scientific working group. *American Journal of Tropical Medicine and Hygiene* 98, 1–49. doi: 10.4269/ajtmh.18-0083

James, S.L., Marshall, J.M., Christophides, G.K., Okumu, F.O. and Nolan, T. (2020) Toward the definition of efficacy and safety criteria for advancing gene drive-modified mosquitoes to field testing. *Vector-Borne and Zoonotic Diseases* 20, 237–251. doi: 10.1089/vbz.2019.2606.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821. doi: 10.1126/science.1225829

Knols, B.G.J. and Bossin, H. (2006) *Identification and Characterisation of Field Sites for Genetic Control of Disease Vectors*. Wageningen UR Frontis Series, Wageningen, Netherlands.

Komen, J, Tripathi, L., Mkoko, B., Ofosu, D.O., Oloka, H. andWangari D. (2020) Biosafety regulatory reviews and leeway to operate: case studies from Sub-Sahara Africa. *Frontiers in Plant Science* 11, 130. doi: 10.3389/fpls.2020.00130

Kormos, A., Lanzaro, G.C., Bier, E., Dimopoulos, G., Marshall, J.M. *et al.* (2020) Application of the relationship-based model to engagement for field trials of genetically engineered malaria vectors. *The American Journal of Tropical Medicine and Hygiene* 104, 805–811. doi: 10.4269/ajtmh.20-0868

Kuwana, Y., Sezutsu, H., Nakajima, K.I., Tamada, Y. and Kojima, K. (2014) High-toughness silk produced by a transgenic silkworm expressing spider (*Araneus ventricosus*) dragline silk protein. *PLoS ONE* 9, e105325. doi: 10.1371/journal.pone.0105325

Lehane, M.J. and Aksoy, S. (2012) Control using genetically modified insects poses problems for regulators. *PLoS Neglected Tropical Diseases* 6(1), e1495. doi: 10.1371/journal.pntd.0001495

Lehmann, P., Ammunét, T., Barton, M., Battisti, A., Eigenbrode, S.D. *et al.* (2020) Complex responses of global insect pests to climate warming. *Frontiers in Ecology and the Environment* 18, 141–150. doi: 10.1002/fee.2160

Lester, P.J., Bulgarella, M., Baty, J.W., Dearden, P.K., Guhlin, J. and Kean, J.M. (2020) The potential for a CRISPR gene drive to eradicate or suppress globally invasive social wasps. *Scientific Reports* 10. doi: 10.1038/s41598-020-69259-6

Li, F., Yamamoto, A., Belikoff, E.J., Berger, A., Griffith, E.H. and Scott, M.J. (2021) A conditional female lethal system for genetic suppression of the global fruit crop pest *Drosophila suzukii*. *Pest Management Science* 77, 4915–4922. doi: 10.1002/ps.6530.

Long, K.C., Alphey, L., Annas, G.J., Bloss, C.S., Campbell, K.J. *et al.* (2020) Core commitments for field trials of gene drive organisms. *Science* 370, 1417 LP–1419. doi: 10.1126/science.abd1908

Mahase, E. (2021) Malaria vaccine becomes first to achieve 75% efficacy goal in trial of children. *BMJ (Clinical Research edn)* 373, n1078. doi: 10.1136/bmj.n1078

McLean, M., Foley, M.-E. and Pehu, E. (2012) *The Status and Impact of Bio Safety Regulation in Developing Economies Since Ratification of the Cartagena Protocol*. Agriculture and Rural Development and Environment departments; joint departmental discussion. Paper No. 3. World Bank, Washington, DC. Available at: https://openknowledge.worldbani.org/handle/10986/18315 (accessed 26 April 2022)

Medlock, J., Luz, P.M., Struchiner, C.J. and Galvani, A.P. (2009) The Impact of Transgenic Mosquitoes on Dengue Virulence to Humans and Mosquitoes. *The American Naturalist* 174, 565–577.

Minde, I.J. and Mazvimavi, K. (2007) The Economics of Biotechnology (GMOs) and the Need for a Regional Policy: the Case for COMESA Countries. In: *AAAE Conference Proceedings*, 377–381. African Association of Agricultural Economists. 2nd International Conference, August 20–22 2007, Accra, Ghana, pp. 25–29.

Mumford, J.D. (2012) Science, regulation, and precedent for genetically modified insects. *PLOS Neglected Tropical Diseases* 6, e1504. doi: 10.1371/journal.pntd0001504

Murphy, B., Jansen, C., Murray, J. and DeBarro, P. (2010) Risk analysis on the Australian release of *Aedes aegypti* (L.) (Diptera: Culicidae) containing *Wolbachia*. *Frontiers in Public Health* 4, 43.

NASEM (2016) *Gene Drives on the Horizon: Advancing Science, Navigating Uncertainty, and Aligning Research with Public Values*. National Academies of Sciences, Engineering, and Medicine. National Academies Press, Washington, DC. doi: 10.17226/23405.

Neuenschwander, P. (1994) Control of the cassava mealybug in Africa: lessons from a biological control project. *African Crop Science Journal* 2, 369–383.

Nickson, T. (2008) Planning environmental risk assessment for genetically modified crops: problem formulation for stress tolerant crops. *Plant Physiology* 147, 494–502.

NRC (1989) *Field Testing Genetically Modified Organisms: Framework for Decisions*. National Research Council. The National Academies Press, Washington, DC. doi: 10.17226/1431

O'Brochta, D.A., Tonui, W.K., Dass, B. and James, S. (2020) A cross-sectional survey of biosafety professionals regarding genetically modified insects. *Applied Biosafety* 25, 19–27. doi: 10.1177/1535676019888047.

Obonyo, D.N., Nfor, L.M. and Uzochukwu, S. (2011) Identified gaps in biosafety knowledge and expertise in Sub-Saharan Africa. *AgBioForum* 14, 71–82.

OECD (2018) *Consensus Document on the Biology of the Mosquito Aedes aegypti*. No. 65 in Series on Harmonisation of Regulatory Oversight in Biotechnology. Organisation for Economic Co-operation and Development, Paris.

OECD (2021) *Chemical Safety and Biosafety Progress Report* No. 41*, June 2021*. Organisation for Economic Co-operation and Development, Paris.

OSTP (1986) Coordinated framework for regulation of biotechnology: announcement of policy and notice for public comment. *Federal Register* 51(123), 23302-50. US Office of Science and Technology Policy, Washington, DC. Available at: https://www.aphis.usda.gov/brs/fedregister/coordinated_framework.pdf (accessed 4 April 2022)

OSTP (1992) Exercise of Federal Oversight Within Scope of Statutory Authority: Planned Introductions of Biotechnology Products into the Environment. *Federal Register* 57(39), 6753–6762. US Office of Science and Technology Policy, Washington, DC.

OSTP (2017) *Modernizing the Regulatory System for Biotechnology Products: Final Version of the 2017 Update to the Coordinated Framework for the Regulation of Biotechnology*. US Office of Science and Technology Policy, Washington, DC. Available at: fda.gov/media/102658/download (accessed 27 April 2020)

Oxitec (2021) *Oxitec receives landmark biosafety approval for New Fall Armyworm control solution*. Available at: https://www.oxitec.com/en/news/oxitec-receives-landmark-biosafety-approval-for-new-fall-armyworm-control-solution

Oye, K.A., Esvelt, K., Appleton, E., Catteruccia, F., Church, G. *et al.* (2014) Regulating gene drives. *Science* 345, 626 LP–628. doi: 10.1126/science.1254287

Pare Toe, L., Barry, N., Ky, A.D., Kekele, S., Meda, W. *et al.* (2021) Small-scale release of non-gene drive mosquitoes in Burkina Faso: from engagement implementation to assessment, a learning journey. *Malaria Journal* 20(1), 1–18. doi: 10.1186/S12936-021-03929-2

Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J. *et al.* (2007) Late-acting dominant lethal genetic systems and mosquito control. *BMC Biology* 5, 11. doi: 10.1186/1741-7007-5-11.

Pureswaran, D.S., Roques, A. and Battisti, A. (2018) Forest insects and climate change. *Current Forestry Reports* 4, 35–50. doi: 10.1007/s40725-018-0075-6

Racovita, M. (2017) Being scientific about socio-economics in GMO decision-making in developing countries. In: Ademola, A., Adenle, E., Morris, J. and Murphy, D.J. (eds) *Genetically Modified Organisms in Developing Countries: Risk Analysis and Governance*. Cambridge University Press, Cambridge, pp. 115–127. doi: 10.1017/9781316585269.011

Roberts, A., De Andrade, P.P., Okumu, F., Quemada, H., Savadogo, M. *et al.* (2017) Results from the workshop 'Problem formulation for the use of gene drive in mosquitoes'. *American Journal of Tropical Medicine and Hygiene* 96, 530–533. doi: 10.4269/ajtmh.16-0726.

Rudenko, L., Palmer, M.J. and Oye, K. (2018) Considerations for the governance of gene drive organisms. *Pathogens and Global Health* 112, 162–181. doi: 10.1080/20477724.2018.1478776

Schairer, C.E., Taitingfong, R., Akbari, O.S. and Bloss, C.S. (2019) A typology of community and stakeholder engagement based on documented examples in the field of novel vector control. *PLoS Neglected Tropical Diseases* 13, e0007863. doi: 10.1371/journal.pntd.0007863

Shelton, A.M., Long, S.J., Walker, A.S., Bolton, M., Collins, H.L. *et al.* (2020) First field release of a genetically engineered, self-limiting agricultural pest insect: evaluating its potential for future crop protection. *Frontiers in Bioengineering and Biotechnology* 7, 482. doi: 10.3389/fbioe.2019.00482

Shepard, D.S., Coudeville, L., Halasa, Y.A., Zambrano, B. and Dayan, G.H. (2011) Economic impact of dengue illness in the Americas. *The American Journal of Tropical Medicine and Hygiene* 84, 200–207.

Simmons, G.S., McKemey, A.R., Morrison, N.I., O'Connell, S., Tabashnik, B.E. *et al.* (2011) Field performance of a genetically engineered strain of pink bollworm. *PLoS ONE* 6, e24110. doi: 10.1371/journal.pone.0024110.

Simoni, A., Hammond, A.M., Beaghton, A.K., Galizi, R., Taxiarchi, C. *et al.* (2020) A male-biased sex-distorter gene drive for the human malaria vector *Anopheles gambiae*. *Nature Biotechnology* 38, 1054–1060. doi: 10.1038/s41587-020-0508-1

Sinkins, S.P. and Gould, F. (2006) Gene drive systems for insect disease vectors. *Nature Reviews Genetics* 7, 427–435. doi: 10.1038/nrg1870

Tabashnik, B.E., Sisterson, M.S., Ellsworth, P.C., Dennehy, T.J., Antilla, L. *et al.* (2010) Suppressing resistance to Bt cotton with sterile insect releases. *Nature Biotechnology* 28, 1304–1307. doi: http://www.nature.com/nbt/journal/v28/n12/abs/nbt.1704.html#supplementary-information

Takken, W., Scott, T.W. and Bogers, R.J. (eds) (2002) *Ecological Aspects for Application of Genetically Modified Mosquitoes*. Proceedings of the Frontis Workshop on Ecological Challenges Concerning the Use of Genetically Modified Mosquitoes for Disease Control, Wageningen, The Netherlands, 26–29 June 2002. Kluwer Academic, Dordrecht, Netherlands.

Teem, J.L., Ambali, A., Glover, B., Ouedraogo, J., Makinde, D. and Roberts, A. (2019) Problem formulation for gene drive mosquitoes designed to reduce malaria transmission in Africa: Results from four regional consultations 2016–2018. *Malaria Journal* 18, 1–13. doi: 10.1186/s12936-019-2978-5

Thizy, D., Emerson, C., Gibbs, J., Hartley, S., Kapiriri, L. *et al.* (2019) Guidance on stakeholder engagement practices to inform the development of areawide vector control methods. *PLoS Neglected Tropical Diseases* 13, e0007286. doi: 10.1371/journal.pntd.0007286

Turner, G., Beech, C. and Roda, L. (2018) Means and ends of effective global risk assessments for genetic pest management. *BMC Proceedings* 12 (Suppl. 8), Article 13. doi: 10.1186/s12919-018-0112-2

UN (1992) *Report of the United Nations Conference on Environment & Development. General Assembly A/Conf.151/26(Vol)1, 12 August 1992.* Available at: https://www.un.org/en/development/desa/population/migration/generalassembly/docs/globalcompact/A_CONF.151_26_Vol.I_Declaration.pdf (accessed 22 April 2022)

UNEP (2005) *Building Capacity for Effective Participation in the Biosafety Clearing-House (BCH) of the Cartagena Protocol on Biosafety*. United Nations Environment Programme, Geneva. Available at: https://wedocs.unep.org/20.500.11822/10002 (accessed 22 April 2022)

USDA (2021) *Unified Website for Biotechnology Regulation*. Available at: https://usbiotechnologyregulation.mrp.usda.gov/biotechnologygov/about (accessed 13 July 2021).

USDA APHIS (2006) Availability of an Addendum to an Environmental Assessment for Field Release of Genetically Engineered Pink Bollworm. *Federal Register* 71(118), 35408–35409. Available at: aphis.usda.gov/brs/fedregister/BRS_20060620a.pdf (accessed 27 April2022)

USDA APHIS (2008) *Use of Genetically Engineered Fruit Fly and Pink Bollworm in APHIS Plant and Pest Control Programs*. Available at: aphis.usda.gov/plant_health/ea/downloads/eis-gen-pbw-ff.pdf (accessed 27 April 2022)

USDA APHIS (2009) Record of Decision: Use of Genetically Engineered Fruit Fly and Pink Bollworm in APHIS Plant Pest Control Programs. *Federal Register* 74, 21314–21316.

van Huis, A. (2020) Insects as food and feed, a new emerging agricultural sector. A review. *Journal of Insects as Food and Feed* 6, 27–44. doi: 10.3920/JIFF2019.0017.

Vargas-Terán, M., Hursey, B.S. and Cunningham, E.P. (1994) Eradication of the screwworm from Libya using the sterile insect technique. *Parasitology Today* 10(3), 119–122.

Vargas-Terán, M., Spradbery, J.P., Hofmann, H.C. and Tweddle, N.E. (2021) Impact of screwworm eradication programmes using the sterile insect technique. In: *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management*, 2nd edn. CRC Press, Boca Raton, Florida, pp. 949–978. doi: 10.1201/9781003035572-29.

Waithaka, M., Belay, G., Kyotalimye, M. and Karembu, M. (2015) Progress and challenges for implementation of the Common Market for Eastern and Southern Africa policy on biotechnology and biosafety. *Frontiers in Bioengineering and Biotechnology* 3, 109. doi: 10.3389/fbioe.2015.00109.

Walker, T., Johnson, P.H., Moreira, L.A., Iturbe-Ormaetxe, I., Frentiu, F.D. *et al.* (2011) The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature* 476, 450–453. doi: 10.1038/nature10355.

WHO (2010) *Progress and prospects for the use of genetically modified mosquitoes to inhibit disease transmission*. Report on planning meeting 1: Technical consultation on current status and planning for future development of genetically modified mosquitoes for malaria and dengue control. WHO, Geneva, 4–6 May 2009. UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases & Foundation for the National Institutes of Health (USA). Available at: https://who.int/iris/handle/10665/44297 (accessed 25 April 2022)

WHO (2017) *The evaluation process for vector control products*. Information note. World Health Organization, Geneva. Available at: apps.who.int/iris/bitstream/handle/10665/255644/WHO-HTM-GMP-2017.13-eng.pdf. (accessed 25 April 2022)

WHO (2018) *Vaccines and immunization: Dengue*. World Health Organization, Geneva. Available at: https://www.who.int/news-room/q-a-detail/dengue-vaccines (accessed 12 July 2021)

WHO (2020) *World Malaria Report 2020*. World Health Organization, Geneva. Available at: https://www.who.int/publications/i/item/9789240015791 (accessed 12 July 2021).

WHO (2021a) *Dengue and severe dengue*. World Health Organization, Geneva. Available at: https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue (accessed 12 July 2021)

WHO (2021b) *WHO recommends groundbreaking malaria vaccine for children at risk*. World Health Organization, Geneva. Available at https://www.who.int/news/item/06-10-2021-who-recommends-groundbreaking-malaria-vaccine-for-children-at-risk (accessed 14 October 2021).

WHO (2021c) *WHO Vector Control Advisory Group (VCAG)*. Available at https://www.who.int/groups/vector-control-advisory-group (accessed 12 July 2021)

WHO/TDR (1991) *Report of the Meeting 'Prospects for Malaria Control by Genetic Manipulation of its Vectors'*. Paper presented at Vector Biology Meeting, Tuscon, Arizona, 27–31 January 1991. TDR/BCV/MAL-ENT/91. WHO Special Programme for Research and Training in Tropical Diseases (Tropical Disease Research (TDR)). World Health Organization, Geneva.

WHO/TDR (2021) *Guidance Framework for Testing of Genetically Modified Mosquitoes*, 2nd edn. World Health Organization (on behalf of the Special Programme for Research and Training in Tropical Diseases (TDR)), Geneva.

WHO/TDR and FNIH (2014) *Guidance Framework for Testing of Genetically Modified Mosquitoes*. 1st edn. World Health Organization, Geneva, and Foundation for the National Institutes of Health. Available at: https://apps.who.int/iris/bitstream/handle/10665/127889/9789241507486_eng.pdf (accessed 16 January 2020)

Wolt, J.D., Keese, P., Raybould, A., Fitzpatrick, J.W., Burachik, M. *et al.* (2010) Problem formulation in the environmental risk assessment for genetically modified plants. *Transgenic Research* 19, 425–436.

Yan, Y. and Scott, M.J. (2020) Building a transgenic sexing strain for genetic control of the Australian sheep blow fly *Lucilia cuprina* using two lethal effectors. *BMC Genetics* 21, Article 141. doi: 10.1186/s12863-020-00947-y

Zanotto, P.M. de A. and Leite, L.C. de C. (2018) The challenges imposed by dengue, zika, and chikungunya to Brazil. *Frontiers in immunology* 9, 1964. doi: 10.3389/fimmu.2018.01964

Zhan, S., Fang, G., Cai, M., Kou, Z., Xu, J. *et al.* (2020) Genomic landscape and genetic manipulation of the black soldier fly *Hermetia illucens*, a natural waste recycler. *Cell Research* 30, 50–60. doi: 10.1038/s41422-019-0252-6.

26 Economics of Transgenic Insects for Field Release

John D. Mumford[1]* and L. Roman Carrasco[2]

[1]*Centre for Environmental Policy, Imperial College London;* [2]*Department of Biological Sciences, National University of Singapore*

26.1 Introduction

The implementation of transgenic insects as components of pest management systems depends on the economic viability of the process in individual cases. The use of radiation-induced sterility in the sterile insect technique (SIT) (see Scott *et al.*, Chapter 17, this volume), inundative biological control releases and live vaccine baits provide some precedents for economic analysis of self-limiting transgenic applications. Classical biological control and the introduction of exotic pollinators provide examples for economic analysis of potential self-sustaining applications of transgenic insects. These examples illustrate several general issues that will affect the economic evaluation of transgenic insect applications, such as: the choice of temporal and spatial dimensions to be used in assessing costs and impacts; assumptions and estimates of uncertainty in projections of costs, performance and impacts; how to capture benefits and attribute costs; and distinctions between public and private roles in implementation.

Two general applications of transgenic insects, self-limiting and self-sustaining, can potentially be implemented as components for pest control programmes (Alphey, 2014; NASEM, 2016; Alphey and Bonsall, 2018; Flores and O'Neill, 2018; EFSA, 2020). Self-limiting forms are either immediately sterile or have inherited lethality that prevents the offspring from maturing, either of which can be used to reduce pest populations in much the same way as SIT using radiation-induced sterility (Dyck and Hendrichs, 2021) (see Scott *et al.*, Chapter 17, this volume). The application of transgenic *Aedes aegypti* with inherited lethality to reduce dengue vector populations is a specific example of this approach using a transgenic technology, with releases having taken place in Brazil following earlier trials in the Cayman Islands and Malaysia and now underway in Florida (Alphey *et al.*, 2011; Harris *et al.*, 2011, 2012; Lacroix *et al.*, 2012; Carvalho *et al.*, 2015; Waltz, 2021) (see Morrison, Chapter 23; Beech *et al.*, Chapter 25, this volume). Considerable work on the economics of SIT has been carried out for the International Atomic Energy Agency (IAEA) and there is a long history of SIT and its economic evaluation (Quinlan *et al.*, 2008; Mumford, 2021). Releases of self-sustaining populations of transgenic insects are also potential applications (Alphey, 2014). These

---

*Corresponding author, email: j.mumford@imperial.ac.uk

self-sustaining populations replace wild insect populations with potential to introduce modified behaviour, physiology, or other properties that reduce their impact as vectors or plant feeders (see Bottino-Rojas and James, Chapter 11, this volume). No examples of this form of transgenic insect application have yet been released.

These two approaches have significant differences in economic terms. While both systems are likely to involve substantial investment in development and regulatory approval, the operating costs are very different. Self-limiting applications require repeated releases and ongoing benefits are directly dependent on continuing operational costs. Furthermore, operations can be stopped very rapidly and alternative control methods could be adopted if circumstances change, without major cost to remove possible residual transgenic populations. Self-sustaining applications require an initial release, or a relatively small number of seeding operations, to establish a transgenic population in the field, from which long-term benefits develop as the released population grows, intensifies and spreads into the wild population, eventually replacing it. As such, post-release costs may be much lower for self-sustaining applications than for continuous self-limiting operations, but it may take some time for the natural population to be replaced and for beneficial impacts to be achieved. In the event that the process needed to be stopped, there may need to be active measures taken to remove the established transgenic population, which could be costly. Transgenic applications of both types are likely to have regulatory surveillance requirements on post-release populations of both the target and some key non-target organisms (EFSA, 2013, 2020), and some capacity to respond to unsatisfactory situations that may arise, from pockets of low performance (as may also occur in conventional management) to possible unexpected negative environmental or health outcomes related to the transgenic element. Area-wide SIT (Dyck and Hendrichs, 2021) and classical biocontrol (Hill and Greathead, 2000; Cooke et al., 2013) are the principal precedents for economic analyses for inundative and inoculative controls, respectively. Some general principles of benefit–cost analysis apply in area-wide pest management, regardless of the control technology. Mumford (2021) addressed issues in relation to SIT, Brown et al. (2019) considered genetic controls, and Kehlenbeck et al. (2012) looked at examples related to several outbreaks of exotic pests in Europe. Preventive release against invasive pests and suppression or eradication of established outbreaks have been important focal purposes for SIT, and classical biocontrol has also mainly been directed at outbreaks of introduced pests. The environmental impact of reducing or removing exotic pest species is widely perceived as beneficial, as they have no natural role in their new environment. Transgenic insect releases may also be directed at invasive species, as has already occurred with *Aedes aegypti* in Brazil, but some other applications could be directed to endemic insects, such as malaria vectors in Africa or fruit flies in Europe, if regulatory approval were given (Alphey, 2014; NASEM, 2016) (see Beech et al., Chapter 25, this volume).

The costs of control include research and development, regulatory approval, implementation operations and training, monitoring, reporting and responses to unintended outcomes. The benefits of area-wide control include (Mumford, 2021; Brown et al., 2019): (i) reduced direct and indirect costs of current control; (ii) reduced losses to health, crops or livestock due to target pests; (iii) reduced environmental impacts from pests and controls; (iv) new market opportunities or improved retention of existing markets for crops or livestock; and (v) greater impetus to invest in areas in which pests have been controlled or health has been improved.

26.2 Inundative Concept

The inundative concept of control involves the continual, regular release of reared organisms to exert some influence on the population of a target species. The target species may be either the same as the species

released, as in the case of sterile insects that mate with wild individuals in the same species, or the target may be another species on which the released organisms have a direct impact, either reducing pest populations, as in biocontrol, or imparting immunity, as in the case of vaccination. Inundative release could be used as a preventive measure, for pest population suppression, or for eradication, and the success of the outcome would be judged against the intended purpose. WHO and IAEA (2020) have recently presented guidelines on inundative releases of sterile insects for vector control that include economic considerations, and Brown *et al*. (2019) also looked at economic issues in inundative releases of insects in genetic control.

Preventive release involves the release of a self-limiting population in an area in which a pest is not generally established. This has been practised in the USA for over 15 years to prevent Mediterranean fruit fly (medfly) outbreaks in California and Florida, with considerable success. The last major introduction prompting major action was related to medfly larvae found in stores after shipment of clementines from Spain to the USA in late 2001. The USA banned imports of Spanish clementines for almost a year, with European estimates that sales of 115,000 tonnes were affected. The principal performance indicators for preventive release programmes are the frequency and scale of pest outbreaks with prevention, compared with the case without prevention (CDFA, 2003). The economic value of preventive release can be estimated by comparing the distributions of outbreak costs, under assumptions of expected frequency and scale over a reasonable time horizon. The cost of continual releases and any marginal additional costs of surveillance would be subtracted from the expected benefit. Surveillance costs may increase with preventive release because of the need to discriminate large numbers of released insects from relatively small numbers of exotic invaders that would indicate an outbreak that would trigger further responsive action. For example, in California, from 1990 to 1996 (prior to preventive release) there were medfly outbreaks in five of the six years, at an annual average cost of US$15.2 million to control; from 1997 to 2002 there were outbreaks in three of the six years at an annual average eradication cost of only US$0.75 million, despite at least 50% more fresh produce imports. The Medfly Exclusion Program was costing US$18.8 million per year during the period 1997–2002, marginally more than the average without preventive release, but with considerably less variability. While control costs on average are slightly higher over this period, there is a significant positive benefit on maintaining trade opportunities for billions of dollars of exports by avoiding the risk that a large outbreak could become a permanently established population, resulting in annual costs estimated at over US$500 million (CDFA, 2003).

No transgenic insects have yet been released in long-term full-scale area-wide pest management programmes; GM sterile *Aedes* mosquitoes were released in commercial development trials in Brazil (and in even smaller scale field tests in several other countries in the western hemisphere) and GM fluorescent-marked *Pectinophthora* moths have been tested in trials in the USA (Simmons *et al*., 2011) (see Scott *et al*., Chapter 17, this volume). However, an oral rabies vaccine based on a transgenic virus (not the rabies virus) in a bait application has been used extensively in the USA, Canada and Europe (Sterner *et al*., 2009), with over 100 million doses distributed since 1989. Baits are applied by air over large areas where wildlife susceptible to rabies occur, so bait costs can be accurately calculated on an average per hectare basis for the areas covered. The more difficult task of estimating the returns on such programmes comes due to the variability of the disease challenge in the area, related to the density of host populations and their mobility, immunity and transmission rates. Key economic indicators included the cost of treatment to humans for rabies following potentially infectious contact with wildlife, reduced vaccination costs for pets, and reduced wildlife culling costs. Because of the complex ecological and epidemiological relationships involved in such control programmes there needs to be a long-term commitment (in this case, greater than 5 years)

to ensure that benefits are realized once the control effort starts. The baiting programmes require greater surveillance and standby management capacity to ensure that effective action can be taken to prevent reintroduction of rabies, which would stop the flow of benefits from control. Benefits were assumed both from the reduction of epidemic intensity in endemic areas and from the prevention or slowing of spread to non-endemic areas. The six studies examined used time horizons ranging from 5 to 40 years. The variability in assumptions and the long-time horizons resulted in very broad ranges of estimated returns, with benefit–cost ratios from 3.4 to 13.1 (Sterner *et al.*, 2009). However, as even the lower end of the range indicates a substantial positive return, the vaccine bait programmes have been widely adopted.

It is therefore essential to be able to capture the complex relationship between pest population reduction and benefits for the economic analysis to proceed. Such relationships may not be linear, with benefits only noticeable when the pest population is at very low levels. In Singapore, for instance, successful vector control reduction of *Ae. aegypti* through habitat destruction is still met with frequent epidemics presumably linked to low levels of herd immunity (Sun *et al.*, 2021). Furthermore, considering that the long-term operating costs of a control programme would be much lower if a near-complete suppression of the pest were attained, aggressive initial releases may often be the most cost-effective option (Undurraga *et al.*, 2016).

Figure 26.1 illustrates a general set of assumptions about time flows of costs and benefits for a long-term inundative control release programme. The longer the time horizon for the project, the more likely it is that net benefits would accrue in this example. However, as the time horizon extends, uncertainties affecting both costs and benefits will increase. For example, over time, conditions may change to reduce the realized benefits. Future costs and benefits need to be expressed as present values, using the concept of future discounting, and this can in part account for the increasing uncertainty of future events. A typical developed country discount rate at present is 3.5% (HM Treasury, 2020), but higher rates may apply in developing countries (national finance ministries provide guidance on rates, but broadly a discount rate would be the local lending interest rate minus inflation, effectively the real rate of interest). Discount rates will also affect the time horizon to realistically consider in an economic analysis. For example, at a discount rate of 10%, benefits 10 years into the future have a present value of less than 40% of their expected nominal future value, and at 20 years into the future the present value is less than 15% of the nominal future value. Longer time horizons at high discount rates add little marginal present value.

An alternative model for inundative releases is for localized releases in response to, or anticipation of, critical outbreaks, which could occur in different places or times (for example, Mains *et al.*, 2019). That kind of programme would have set-up costs like long-term release programmes (dependent on the anticipated capacity needed) but may be expected to have much greater uncertainties over operating costs and benefits over the following years. Despite this, the flexibility to provide control at relatively short notice may make such a programme economically attractive as a risk mitigation approach.

For inundative releases, an important issue is how to ensure that the benefits of the released insects are captured within the boundaries of the management zone. It is desirable for the management zone to be selected with a high density of potential valuable hosts affected by the pest to be controlled to obtain a good return from the control effort. To manage reinvasion or immigration into the managed zone, a buffer treatment area may need to surround the zone, which adds to the cost without getting full or even partial benefits. Management zones should therefore be carefully selected to ensure maximum potential benefits within the zone, while minimizing the buffer treatment areas around the perimeter. Some trade-off may occur between an area designed with more streamlined buffer zones but with less potential efficiency from
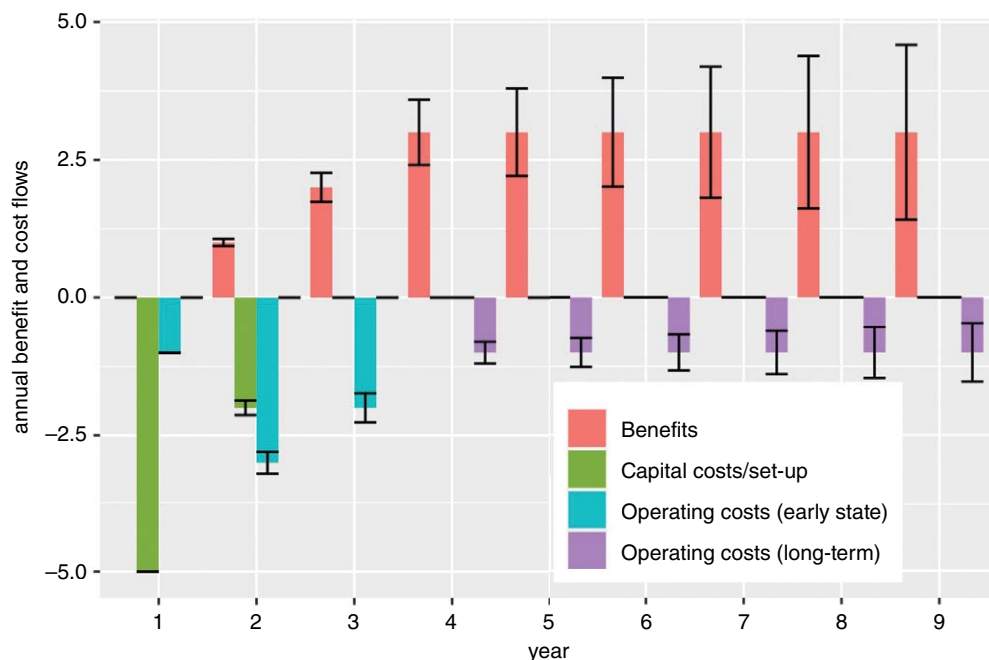
Fig. 26.1. Time horizons. A fundamental issue in assessing the net benefit of a transgenic insect release programme is the time horizon for the costs and benefits, with capital costs (such as research, development, regulatory approval) at the start and benefits evolving after operational costs are incurred. Operating costs (long-term) are depicted as constant over time but could decrease as the target pest population decreases. Uncertainty increases over time and in proportion to costs or benefits, depicted as error bars.

a more heterogeneous management area within the boundary.

Large-scale inundative release programmes may need to occur in a series of zones progressing in annual increments, simply because of the scale of releases required. An example is the SIT eradication programme for the New World screwworm that has pushed the infested area from the USA south to Panama, and the medfly from the USA to southern Mexico (Dyck and Hendrichs, 2021) (see Scott *et al.*, Chapter 17, this volume). In those cases, it is logical to move the zones south from the USA for ecological reasons, but the pace and direction of movement of control zones may also be determined by the order of potential inputs and benefits (Brown *et al.*, 2019; Mumford, 2021). Early benefits are important to achieve an overall net gain from a control programme with high initial investment costs. Eradication following a progressive

wave of inundative releases can give high levels of benefits, particularly as trade opportunities open up with reduced quarantine restrictions, but eradication brings with it the added cost of surveillance to prove pest-free status and standby control capacity to maintain it.

Regulatory costs are likely to be a significant additional cost in transgenic insect implementation compared with radiation-induced sterile insect release. In the case of radiation-induced sterility, the release of the insects is now a long-established practice in many countries and is used both for control of insect populations already present and for preventive control of potential invasions. When radiation is used to induce sterility, SIT regulators have repeatedly accepted that the insects released, as a population, are unable to effectively reproduce even if the released population is not quite 100% sterile and they do not pose

an unacceptable risk to the environment or human health. However, transgenic insects fall into a separate class that requires regulation because they are widely perceived or designated in law as being inherently different from wild populations (EFSA, 2013) (see Beech *et al.*, Chapter 25, this volume). Direct regulatory costs and time delays in implementation while regulatory approval is considered would both have negative effects on the net benefits of transgenic insect releases, particularly if programmes have relatively short time horizons. Regulatory uncertainty in particular markets is also likely to discourage private investment in transgenic insect applications. Regulatory approval of releases will specify standard operating procedures for releases, which will impose management costs and quality control costs on the programme, which may vary depending on the stringency of the procedures demanded. Compared with non-transgenic releases, additional post-release monitoring is likely to be imposed for case-specific surveillance, to demonstrate that risk assumptions on transgenic applications were correct, and some level of general surveillance to identify and respond to possible unanticipated negative outcomes (EFSA, 2013, 2020). This also occurs in many countries for the regulation of new organisms for classical biological control introductions.

Economic analysis may also be used to determine pricing for SIT inputs, such as reared insects, which would be as applicable for transgenic insects as for irradiated insects. The tool presented by Quinlan *et al.* (2008) incorporates estimates of cost distributions for the range of rearing inputs for sterile insects, allowing for quality factors, colony maintenance and the scale and time horizon of releases. The output is an estimated distribution of capital and variable costs related to production over a specified time period, from which appropriate prices or budgets can be taken. Their analysis of production facility costs, specifically for sterile medfly (the most common large-scale rearing example), for a range of weekly capacity levels indicated that there are limited economies of scale with larger facilities. The risks associated with equipment or management failures in large facilities mean that duplicate production lines are essential, which make up for some economies of scale. Reducing production costs, while still maintaining high quality (levels of sterility, proportion of males and mating compatibility), is important to keep reared insect prices low enough to compete with other forms of control. However, the cost of the reared insects is only one component of the overall costs. Releasing insects efficiently and monitoring the process are also very significant costs of an inundative release programme.

Another key decision is the location of the facilities for the different stages of the insect production process. For instance, egg production can occur in centralized large facilities in countries with low labour and land costs, with the eggs being shipped to facilities for mosquito rearing near the release location (Undurraga *et al.*, 2016). Centralized production, however, may not be feasible in cases where locally introgressed strains are needed for release. The quality of the introgressed strains to adapt to the local environment and mate with wild insects will determine their efficacy and influence the numbers to be released and eventual costs.

26.3 Inoculative Concept

The inoculative concept of control is based on self-sustaining populations intended to replicate and spread, with the expectation that they will interact with other organisms in a beneficial manner, either reducing a target population of a different species through predation, parasitism or disease, or replacing a wild population of the same species with a new one with more desirable attributes. The release of self-sustaining transgenic insects for pest control presents analogies with classical biocontrol (Brown *et al.*, 2019) and release of self-sustaining populations of *Wolbachia* infected insects (O'Neill *et al.,* 2018). There are also substantial differences: instead of using a different organism related to the pest, for instance through predation or parasitism, the released agent is the same species as the target pest. Transgenic insect

release is especially relevant to insects that are vectors of diseases, for example *Anopheles* mosquitoes for malaria and *Aedes* mosquitoes for dengue, and involves the release of insects incapable of sustaining disease transmission (if female mosquitoes are released).

Self-sustaining transgenic insects attain disease control by means of population replacement through gene drive by which a gene of interest is spread through successive generations from the released insects into the native population. Transgenic and non-GM gene drive systems have been proposed to introduce disease refractory genes into a population: transposable elements, meiotic drive genes, homing endonuclease genes, engineered under-dominance and *Wolbachia* (Sinkins and Gould, 2006). Each system leads to different mosquito fitness costs, female fecundity, mating competitiveness, effectiveness to spread the gene more rapidly than Mendelian inheritance and the necessary release numbers. For instance, whereas engineered under-dominance can drive transgenes to high and stable frequencies but requires large initial releases, *Wolbachia* and meiotic drive cannot maintain high frequency of transgenes but can drive the transgene from initial low release numbers (Huang *et al.*, 2007). These differences in release numbers and the power of the gene drive mechanism play a fundamental role in the time needed to reduce transmission, if at all, which is a key factor in the cost-effectiveness of each mechanism.

Although the dynamics of transgenic insect release and potential spread through a native population have been studied (North *et al.*, 2019; North *et al.*, 2020), little is known about the implications for control costs and the benefits of avoided costs from different types of release and gene drive strategies. The cost-effectiveness of the intervention is inherently conditioned by the success of the gene drive mechanisms, thus compensating in later years for the initial investment in the development of the technology (see Fig. 26.1). The releases of self-sustaining *Wolbachia*-infected *Ae. aegypti* mosquitoes for dengue control offer the nearest examples for future gene drive release

programmes (Brady *et al.*, 2020). These programmes are focused on dense urban environments; conditions for control in rural areas with low human population densities and more diverse environmental quality are very different.

Because knowing the possible biological and epidemiological outcomes of different interventions is necessary to estimate the cost-effectiveness, informing policy decisions would require coupling population genetics and epidemiological models with cost–benefit analyses. For instance, given the behaviour of the gene drive system, the distribution of potential cost-effectiveness is expected to be bimodal, depending on the success or failure of the programme. This bimodality responds to the unstable equilibrium of the system in which there exists a release threshold, above which the refractory gene invades the local population and below which the refractory gene is driven to extinction (Davis *et al.*, 2001). North *et al.* (2019, 2020) demonstrated the different impacts through a modelled release of a proposed gene drive *Anopheles* strain for malaria control across a large area in West Africa. Heterogeneous environmental conditions (seasonality and connectedness) resulted in very different outcomes in their modelled performance, with favourable aseasonal and connected environments showing high potential performance, while, in highly seasonal unconnected areas, the likelihood of collapse of the transgenic population was high in subsequent seasons. Inevitably, gene drive systems would be expected to spread well through favourable environments but are likely to reach areas with conditions that limit their establishment and spread when they are released on a continental scale.

The cases of *Aedes* and *Anopheles* self-sustaining inoculative systems demonstrate very different release, management and performance strategies. *Ae. aegypti* is primarily an urban vector and release and subsequent spread is required within relatively homogeneous urban environments. In addition, the capacity of *Aedes* eggs to survive desiccation offers stability over dry seasons. *Anopheles* spp. transmitting malaria

occur in diverse rural areas and natural mosquito populations can be severely challenged in dry seasons, which may be a problem for the sustainability of gene drives with fitness costs affecting dry season survival or recolonization. The logistics of release are less expensive in limited urban areas with easy transport infrastructure and post-release monitoring and other management can be carried out more efficiently than in widespread rural areas. For *Anopheles* control the most marginal areas, in terms of environmental suitability, are likely to be the least accessible, since human population density will be low and associated infrastructure will also be poor. Large proportions of the rural areas will have very low populations of anthropophilic mosquitoes, which adds to the costs per area while reducing area-based benefits. Alternative mosquito release methods, such as the use of drones, as demonstrated in Brazil, have the potential to substantially reduce the release costs of area-based programmes, making them more feasible in rural areas (Bouyer *et al*., 2020).

The economic cost-effectiveness modelling approach taken by Brady *et al*. (2020) for *Wolbachia*-infected *Aedes* in Indonesia considers four phases of a programme: (i) set-up (development, rearing, baselines); (ii) release (based on human population density as a proxy for mosquito population density); (iii) early post-release monitoring (to check on establishment and organize additional releases as needed); and (iv) later post-release monitoring (to check on longer-term equilibrium populations and indications of performance over time). Similar phases could be expected in a self-sustaining gene drive system. Brady *et al*. (2020) demonstrated that the pace of release affects both costs and benefits. Faster release costs more but brings on benefits earlier, which is more valuable. They also highlighted that the time-frame for an economic analysis is an important factor, since the present value is increased over longer horizons by additional cumulative benefits at relatively low marginal cost. Their 10-year timeframe is based on evidence of sustained population impacts from *Wolbachia*-infected *Aedes* released in Australia over that period, and is in line with

similar timeframes used in analyses of inundative releases (Mumford, 2021).

Metchanun *et al*. (2022) modelled the cost-effectiveness of a theoretical self-sustaining gene drive *Anopheles* system in the Democratic Republic of Congo (DRC). Because the DRC covers a large and diverse area, the cost-effectiveness model is based on a set of representative areas in which cost, performance, malaria prevalence and current management parameters can be taken from local evidence. The benefits of replacing current management are determined by an analysis that estimates the conditions under which the gene drive performance makes those management inputs marginally less cost-effective.

It has sometimes been considered uneconomical to release large numbers of transgenic mosquitoes for population replacement at a large scale (Sinkins and Gould, 2006). However, these recommendations are based on biological models and might vary considerably if the different costs and benefits are computed. The practicality of rearing large numbers of insects has been demonstrated for a number of species in SIT programmes and costs have fallen considerably with experience and research on more efficient methods (Dyck and Hendrichs, 2021). While fixed and sunk costs such as capital, technology development and regulatory expenditures are expected to be considerable, variable costs like rearing and releasing transgenic insects may be substantially lower than the potential avoided costs due to disease burden reductions, thus justifying the release of large numbers of insects. This is due to the high impact of diseases in humans.

For instance, in the case of dengue in Singapore, annual costs due to hospitalization, outpatient treatment and job absenteeism amount to US$50 million (2010 values) while another US$50 million are spent annually on vector control and mosquito habitat destruction (Carrasco *et al*., 2011). These potentially avoided costs in the later years of a programme are several orders of magnitude greater than conservative assumptions for variable costs, such as rearing and release costs, especially if a small threshold

of release of 1:300 transgenic to native mosquito is needed (Davis *et al*., 2001). Considering the large differences in avoided costs between unsuccessful and successful outcomes, releasing a number of mosquitoes several orders of magnitude above the necessary threshold to guarantee population replacement, or gene drive, could still be a cost-effective intervention.

Spatial social and environmental heterogeneities further increase the complexity of the cost–benefit analysis due to an increase in the uncertainty of the effectiveness of the programme. North *et al*. (2020) demonstrated simulations on gene drives against malaria vectors in West Africa accounting for spatial heterogeneities, which suggested great variability in outcomes. In favourable conditions they showed a modelled potential reduction of the vector populations of around 95% after only 4 years (North *et al.,* 2020), but in unfavourable areas there may often be no control effect in some locations. Such variability could be mitigated by additional releases, but at some extra cost of monitoring and release operations. In addition, for large-scale programmes, the presence of mountain ranges, rivers or even highways (Hemme *et al*., 2010) might represent important reproductive barriers. Imperfect mixing, in turn, might imply a substantial delay in the replacement of the population, which would have important implications on the cost-effectiveness of the programme by not capturing the substantial avoided costs due to disease reduction in the early years. Localized high levels of infection could reduce the opportunities to save other preventive costs because of the risks of these areas extending to areas already under partial control.

In addition, the spatial and temporal scales of the release strategy need to be adequate for the programme to be cost-effective. Frequent small releases in time have been shown to be adequate to match native population fluctuations according to random mating models compared with spatially aggregated pattern releases (Kiszewski and Spielman, 1998). However, frequent releases are more expensive than less frequent, larger releases for the same total numbers; and more dispersed releases are more expensive than aggregated releases with the same total numbers. Subsequent monitoring costs may also be affected by the temporal and spatial patterns of release. Heterogeneities in the landscape require spatially explicit release models (e.g., Magori *et al*., 2009; North *et al*., 2020; Metchanun *et al*., 2022) that incorporate dispersal and mating to identify the most cost-effective release strategies.

From an economic perspective, self-sustaining populations present several differences compared with self-limiting approaches (Table 26.1). The main advantage is that self-sustaining interventions may not require continual or periodic releases after the population has been replaced, thus averting the long-term variable costs due to release and rearing of the transgenic insects. The main disadvantages occur if the initial functional mechanism within the transgenic insects, which provides the beneficial effect of replacement, is overcome through the evolution of any form of resistance in the vector or pathogen, negating the avoided costs from disease reduction. As a result, the flow of benefits in the later years of the time horizon would fail. In such a case, there may be additional development and regulatory costs needed to develop alternative transgenic applications, and one might envision a series of interventions and a fluctuating level of benefits and public interest. A series of interventions with relatively short-term benefits may still have a net benefit as long as additional development and regulatory costs are low enough.

In addition, self-sustaining programmes might present longer delays in implementation due to more demanding regulatory approval. In contrast, because self-sustaining populations might be capable of spreading to large populations from initially small releases, they may be considered to be more advantageous in low-income settings because the continuity of the programme is not jeopardized by funding instability and the logistic requirements of releases are not so high as in continual inundative release programmes.

Self-sustaining interventions are not necessarily mutually exclusive with SIT

Table 26.1. A comparison of economic aspects of self-limiting and self-sustaining transgenic insects for pest control.

Aspect	Self-limiting	Self-sustaining	Economic implications
Temporal release	Periodic and maintained even after population suppression	Not needed once the gene has been fixed	Self-sustaining is economically more attractive
Spatial release	Good mixing is important for effectiveness	Good mixing might be important for rapid results	Self-sustaining more attractive if obtaining results is not a short-term objective
Externalities	Not relevant unless within dispersal distance of one generation	Might create positive or negative externalities	Provision of funds for liability for self-sustaining insects, reverse programme needed. Self-limiting more attractive
Factory	Facilities for rearing needed for time span of the project	Facilities not needed or temporary	Self-sustaining economically more attractive
Resistance emergence	In theory not an issue	Could emerge	Intervention might not be effective after a few years, self-limiting more attractive
Logistics needs	Continued need	Peak of needs at the beginning of the project	Self-sustaining more attractive but difficult to engage private business
Need to revert spread	Not an issue	Necessary to have contingency plans	Self-limiting is more attractive
Regulatory approval	Self-limiting nature might facilitate approval	Might delay the start of the programme	Delays might discourage private business. Self-sustaining less attractive

methods or traditional vector control methods and vaccines. The implementation of multiple methods might be synergistic. For instance, population suppression through either insecticides or SIT might be necessary prior to population replacement. On the other hand, vaccines may be necessary to increase herd immunity and avoid spikes of transmission through 'rebound effects' if resistance to the refractory gene emerges (Scott *et al.*, 2002).

Spatio-temporal international coordination of releases is essential to attain large-scale suppression and avoid reintroductions in regions with low herd immunity (Scott *et al.*, 2002). An example of continental successful coordination for biocontrol of an agricultural pest was the release of the parasitic wasp *Epidinocarsis lopezi* that fed off the mealybug pest of cassava in Africa (Nweke *et al.*, 2000; Nweke, 2009). The mealybug was accidentally introduced from South America and after 10 years the spread of the pest threatened cassava production

throughout Africa. One of the keys of the success of the programme was to allocate coordination responsibilities to an international agency, the International Institute of Tropical Agriculture (IITA). IITA carried out mass rearing, distribution and release of the wasp with collaborators in each country. The programme involved 30 countries and 120 release sites, but a central agency contributing to efficient rearing, release and monitoring kept quality high and costs lower than if the programme was fully decentralized (Zeddies *et al.*, 2001). As a result, yield losses were reduced by 2.5 t/ha and benefit–cost ratios of 149 to 1 were estimated for the programme (Nweke *et al.*, 2000). More limited and less expensive introduction could eventually have achieved continental impact through natural spread, but the benefits would have occurred more slowly, resulting in much lower benefit–cost ratios in present value terms.

Cooke *et al.* (2013) considered the long-term benefits of classical biological

control for feral rabbits in Australia since 1950. The estimated direct benefits were associated with increased production and indirect benefits were related to reduction or replacement of previous rabbit control methods. They also commented on the inability to rule out alternative development pathways once classical biological control was in place, citing the case of failed biological control of rabbits in New Zealand which was followed by adoption of alternative satisfactory controls. So, in estimating long-term benefits, the potential for other control opportunities arising should also be noted. This is a further indication that relatively short time horizons for benefits should be used, say 10–15 years, to avoid the uncertainty inherent in broader technological innovation.

Similar approaches might be needed for the implementation of large-scale self-sustaining transgenic insect release programmes. International coordination would be needed not only to increase the odds of success but, if unexpected negative effects occur, to mitigate negative externalities that could be borne by neighbouring countries. On the other hand, international coordination is also needed to prevent the creation of positive externalities that act as an incentive for neighbouring countries to be free-riders in a programme and wait for the technology to be paid for and adopted elsewhere.

26.4   Funding Investment and Capturing Economic Benefits

An important issue in any consideration of the economics of transgenic insect implementation is how to capture the benefits of the releases in a way that can be used to offset the implementation costs to the institutions that provide the funds. Classical biocontrol is mainly carried out by governments (Hill and Greathead, 2000), so that public funds are used to deliver broad social benefits. An example of a privately funded self-sustaining release programme is in the related field of pollinator release. The weevils that pollinate oil palm in its native range were collected in West Africa and released, after some official quarantine, in several Southeast Asian countries (Syed *et al.*, 1982) to replace the expensive and inefficient hand pollination needed to develop the crop in a region without natural pollinators. The large plantation companies gained most directly from their investment in the self-sustaining pollinator releases. Beyond the larger plantation companies, there were also smallholders who were free-riders in the programme. However, the costs to the industry were relatively small, and the larger companies also benefited from greater production by smallholders through their interests in processing. An example of private investment in SIT has occurred in Canada, for codling moth control (Bloem *et al.*, 2007). A subscription scheme, organized by the local fruit industry, operated the rearing and release programme. Public–private partnerships could also provide funds for programmes with mixed private and public gains.

Demonstrating effectiveness of self-sustaining interventions may be challenging, especially given the cyclic nature of epidemics and large natural fluctuations of some pest populations, such as mosquitoes. If self-sustaining interventions are further combined with other methods, teasing out the effect of each approach might prove very complex through data analysis alone. In some cases, a series of interventions may be needed as prerequisites to bring the pest and host populations to levels low enough for controls such as SIT to become feasible (e.g., Kovaleski and Mumford, 2007). Considering the potential, interactions between multiple interventions, particularly density-dependent factors that affect control performance, would be necessary.

In contrast to SIT methods, self-sustaining populations might not represent as attractive a business venture for private companies since, on top of the regulatory and monitoring costs, revenues through transgenic insect sales may not continue through time. Return on investment may need to come through licensing the product prior to open release. Liability for any harm may need to be passed to the licensee, which may also affect the immediate value of a licence,

unless governments support limited potential liabilities if unintended impacts occur (Table 26.1).

26.5 Capturing Public Health Benefits

One of the most relevant applications of transgenic insects is the control of vector-borne diseases. In contrast to the economic evaluation of agricultural pest control projects, public health projects involve the estimation of disease burden reductions. Public health agencies need to be able to compare different health projects to allocate their resources more efficiently and effectively. The comparison of public health interventions is attained through cost-effectiveness ratios (CERs) that involve a measure of the net cost of the project per unit of reduction of disease burden:

$$CER = \frac{C_p - C_s}{A_{DALY}}$$

where C_p is the net present value of the cost of the project (regulatory costs, release costs, monitoring, etc.), C_s represents the net present value of costs avoided due to the project, and A_{DALY} denotes the net present value of the averted disease burden (after comparing disease burden with and without the programme). Avoided costs are very different to those of agricultural projects and their estimation involves the use of techniques from health economics such as human-capital or friction-cost methods. Avoided costs would represent direct costs such as reduced hospitalization costs and indirect costs such as reduced losses of productivity to the economy due to reduced job absenteeism.

A standard metric for disease burden reductions that combines the life-years lost due to death, ill-health or disability, and thus capable of integrating mortality and morbidity, is disability-adjusted life years (DALYs) (Murray, 1994). One DALY is equivalent to the value of a lost year of a normal healthy life for the population affected. Expressing the cost-effectiveness of transgenic insect interventions as net cost per DALY averted allows the comparison of the projects with currently adopted health interventions. The cost-effectiveness of transgenic insect releases will also depend on the available resources and the urgency of other public health problems competing for resources in each country. Given the high economic and health impacts of vector-borne diseases and the potential of transgenic insects to reduce transmission substantially, both self-limiting and self-sustaining transgenic releases might be cost-saving technologies, for which the potential avoided costs are higher than the costs of the intervention leading to negative CER. The social benefits of public health interventions, compared with agricultural pest control, might also encourage governments to subsidize development and implementation of new transgenic technologies.

The estimation of cost-effectiveness ratios might be complex, as it requires coupling epidemic models to estimate the disease burden reductions (DALYs averted) due to transgenic insect release (Alphey et al., 2011) and cost–benefit analysis to identify the costs of the project and the potential avoided costs (Carrasco et al., 2011). Cost-effectiveness analyses for transgenic mosquito releases are currently very scarce (an exception using agent-based models is Metchanun et al., 2022). Their development could be a way to demonstrate the potential of the technology to policy makers, thus facilitating its acceptance and adoption.

26.6 Conclusions

There are many precedents for economic analysis of pest control based on reared and released insects, which depend mainly on the intended purpose of the releases. Transgenic insect applications are inherently similar in principle to SIT and classical biocontrol, as examples of self-limiting and self-sustaining beneficial insect release concepts. Self-limiting transgenic insects and radiation SIT are most closely related, because they both involve the release of an insect

population that primarily affects the same species through competitive mating. Classical biocontrol involves an interaction with another species, as a predator, parasite, competitor or pathogen, while self-sustaining transgenic insects could express benefits through altered behaviour or physiology that affected another species of interest, such as a vectored pathogen. Self-sustaining genetic control could also be aimed at suppression of the wild target population of the same species, unlike classical biological control. The inclusion of a transgenic element in the insects released introduces additional regulatory costs prior to introduction, because of the general concerns about transgenic releases into the environment. Additional regulatory costs could be significantly affected by the type of transgenic element, a genetic marker may require less expensive justification than a more active element, and transgenic elements that have already been used in the field would be less costly to approve than those without precedents. While genetic modification brings regulatory requirements, there are also significant regulatory issues around releases of any exotic insects in many countries. There may also be additional production costs because of quality control needed to ensure specific genetic qualities in the mass rearing, and there may be additional post-release surveillance and responsive capacity requirements because of the transgenic element. However, quality control is important for ensuring continued good performance in most area-wide management programmes. The numerous examples of cost–benefit analyses applied to SIT and classical biocontrol demonstrate that efforts to achieve early benefits are essential to ensure high returns.

Cost–benefit analysis must recognize boundaries in time and space. The more limited these are, the less uncertainty will arise in the analysis. However, shorter and narrower estimates reduce the potential for higher net benefits, assuming there are significant initial implementation costs, which will no doubt arise in developing the technology and gaining regulatory approval. Stochastic models of benefits and costs allow uncertainty to be included in the analysis explicitly, so that risks related to extent of the pest or disease challenge, control performance and market values can be considered.

The time involved in obtaining regulatory approval for transgenic insects may be a significant constraint in realizing benefits from the technology. As the technology itself is evolving rapidly, there may be cases where regulations as currently in place are not appropriate to some of the novel technologies. Recent cases have demonstrated the difficulty of regulators even to determine their authority to regulate novel applications that do not immediately fit the descriptions of existing regulations, such as the release of *Wolbachia*-infected insects or the application of gene editing technologies in releases.

The most likely business models appear to be public investment for the public good, as has been the case for most SIT and classical biocontrol. However, private investment may be recovered through licensing self-sustaining applications, or providing rearing, release and monitoring services for self-limiting applications.

References

Alphey, L. (2014) Genetic control of mosquitoes. *Annual Review of Entomology* 59, 205–224.
Alphey, N. and Bonsall, M.B. (2018). Genetics-based methods for agricultural insect pest management. *Agricultural and Forest Entomology* 20, 131–140. doi: 10.1111/afe.12241
Alphey, N., Alphey, L. and Bonsall, M.B. (2011) A model framework to estimate impact and cost of genetics-based sterile insect methods for dengue vector control. *PLoS One* 6, e25384.
Bloem, S., Carpenter, J., McCluskey, A., Fugger, R., Arthur, S. and Wood, S. (2007) Suppression of the codling moth *Cydia pomonella* in British Columbia, Canada using an area-wide integrated approach with an SIT component. In: Vreysen, M.J.B., Robinson, A.S. and Hendrichs, J. (eds) *Area-wide Control of Insect Pests: From Research to Field Implementation*. Springer, Dordrecht, the Netherlands, pp. 591–601.

Bouyer, J., Culbert, N.J., Dicko, A.H., Pacheco, M.G., Virginio, J. *et al.* (2020) Field performance of sterile male mosquitoes released from an uncrewed aerial vehicle. *Science Robotics* 5(43), eaba6251

Brady, O.J., Kharisma, D.D., Wilastonegoro, N.N., O'Reilly, K.M., Hendrickx, E. *et al.* (2020) The cost-effectiveness of controlling dengue in Indonesia using *wMel Wolbachia* released at scale: a modelling study. *BMC Medicine* 18, 186. doi: 10.1186/s12916-020-01638-2

Brown, Z.S., Jones, M. and Mumford, J.D. (2019) Economic principles and concepts in area-wide genetic pest management. In: Onstad, D. and Crain, P. (eds) *The Economics of Integrated Pest Management of Insects*. CAB International, Wallingford, UK, pp. 96–121.

Carrasco, L.R., Lee, L.K., Lee, V.J., Ooi, E.E., Shepard, D.S. *et al.* (2011) Economic impact of dengue illness and the cost-effectiveness of future vaccination programs in Singapore. *PLoS Neglected Tropical Diseases* 5, e1426.

Carvalho, D.O., McKemey, A.R., Garziera, L., Lacroix, R., Donnelly, C.A. *et al.* (2015) Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. *PLoS Neglected Tropical Diseases* 9, e0003864.

CDFA (2003) Preventing biological pollution: the Mediterranean fruit fly exclusion program. California Department of Food and Agriculture, Sacramento, California.

Cooke, B., Chudleigh, P., Simpson, S. and Saunders, G. (2013) The economic benefits of the biological control of rabbits in Australia, 1950–2011. *Australian Economic History Review* 53, 91–107.

Davis, S., Bax, N. and Grewe, P. (2001) Engineered underdominance allows efficient and economical introgression of traits into pest populations. *Journal of Theoretical Biology* 212, 83–98.

Dyck, V.A. and Hendrichs, J. (eds) (2021) *Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management*. CRC Press, New York, pp. 731–752. doi: 10.1201/9781003035572

EFSA (European Food Safety Authority) (2013) Guidance on the environmental risk assessment of genetically modified animals. *EFSA Journal* 11(5), 3200.

EFSA (2020) Scientific Opinion on the adequacy and sufficiency evaluation of existing EFSA guidelines for the molecular characterisation, environmental risk assessment and post-market environmental monitoring of genetically modified insects containing engineered gene drives. *EFSA Journal* 18(11), 6297. doi: 10.2903/j.efsa.2020.6297

Flores, H.A. and O'Neill, S.L. (2018) Controlling vector-borne diseases by releasing modified mosquitoes. *Nature Reviews Microbiology* 16, 508–518.

Harris, A.F., Nimmo, D., McKemey, A.R., Kelly, N., Scaife, S. *et al.* (2011) Field performance of engineered male mosquitoes. *Nature Biotechnology* 29, 1034–1037.

Harris, A.F., McKemey, A.R., Nimmo, D., Curtis, Z., Black, I. *et al.* (2012) Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nature Biotechnology* 30, 828–830.

Hemme, R.R., Thomas, C.L., Chadee, D.D. and Severson, D.W. (2010) Influence of urban landscapes on population dynamics in a short-distance migrant mosquito: evidence for the dengue vector *Aedes aegypti*. *PLoS Neglected Tropical Diseases* 4, e634.

Hill, G. and Greathead, D. (2000) Economic evaluation in classical biological control. In: Perrings, C., Williamson, M.H. and Dalmazzone S. (eds) *The Economics of Biological Invasions*. Edward Elgar Publishing, Cheltenham, UK, pp. 208–223.

HM Treasury (2020) *The Green Book*. HM Treasury, London, UK.

Huang, Y., Magori, K., Lloyd, A.L. and Gould, F. (2007) Introducing transgenes into insect populations using combined gene-drive strategies: modeling and analysis. *Insect Biochemistry and Molecular Biology* 37, 1054–1063.

Kehlenbeck, H., Cannon, R., Breukers, A., Battisti, A., Leach, A. *et al.* (2012) A protocol for analysing the costs and benefits of plant health control measures. *EPPO Bulletin* 42, 81–88.

Kiszewski, A. and Spielman, A. (1998) Spatially explicit model of transposon-based genetic drive mechanisms for displacing fluctuating populations of anopheline vector mosquitoes. *Journal of Medical Entomology* 35, 584–590.

Kovaleski, A. and Mumford, J.D. (2007) Pulling out the evil by the root: the codling moth eradication program in Brazil. In: Vreysen, M.J.B., Robinson, A.S. and Hendrichs, J. (eds) *Area-Wide Control of Insect Pests: From Research to Field Implementation*. Springer, Dordrecht, Netherlands, pp. 581–590.

Lacroix, R., McKemey, A.R., Raduan, N., Lim, K.W., Wong, H.M. *et al.* (2012) Open field release of genetically engineered sterile male *Aedes aegypti* in Malaysia. *PLoS ONE* 7, e42771.

Magori, K., Legros, M., Puente, M.E., Focks, D.A., Scott, T.W. *et al.* (2009) Skeeter Buster: a stochastic, spatially explicit modeling tool for studying *Aedes aegypti* population replacement and population suppression strategies. *PLOS Neglected Tropical Diseases* 3(9), e508.

Mains, J.W., Kelly, P.H., Dobson, K.L., Petrie, W.D. and Dobson, S.L. (2019) Localized control of *Aedes aegypti* (Diptera: Culicidae) in Miami, FL, via inundative releases of *Wolbachia*-infected male mosquitoes. *Journal of Medical Entomology* 56, 1296–1303.

Metchanun, N., Borgemeister, C., Amzati, G., von Braun, J., Nikolov, M. *et al.* (2022) Modeling impact and cost-effectiveness of gene drives for malaria elimination in the Democratic Republic of the Congo. *Evolutionary Applications* 15(1), 132–148. doi: 10.1111/eva.13331

Mumford, J.D. (2021) Design and evaluation of programmes integrating the sterile insect technique. In: Dyck, V.A. and Hendrichs, J. (eds) *Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management*. CRC Press, New York, pp. 731–752. doi: 10.1201/9781003035572

Murray, C.J.L. (1994) Quantifying the burden of disease – the technical basis for disability-adjusted life years. *Bulletin of the World Health Organization* 72, 429–445.

NASEM (2016) *Gene Drives on the Horizon: Advancing Science, Navigating Uncertainty, and Aligning Research with Public Values*. *National Academies Press, Washington, DC.* doi: 10.17226/23405

North, A.R., Burt, A. and Godfray, H.C.J. (2019) Modelling the potential of genetic control of malaria mosquitoes at national scale. *BMC Biology* 17, 26.

North, A.R., Burt, A. and Godfray, H.C.J. (2020) Modelling the suppression of a malaria vector using a CRISPR-Cas9 gene drive to reduce female fertility. *BMC Biology* 18, 98.

Nweke, F. (2009) *Controlling cassava mosaic virus and cassava mealybug in Sub-Saharan Africa. IFPRI Discussion Paper 00912*, International Food Policy Research Institute, Washington, DC.

Nweke, F.I., Lutete, D., Dixon, A.G.O., Ugwu, B.O., Ajobo, O. *et al.* (2000) *Cassava production and processing in the Democratic Republic of Congo*. Collaborative Study of Cassava in Africa, Crop Research Institute, Democratic Republic of Congo and International Institute of Tropical Agriculture, Ibadan, Nigeria.

O'Neill, S.L., Ryan, P.A., Turley, A.P., Wilson, G., Retzki, K. *et al.* (2018) Scaled deployment of *Wolbachia* to protect the community from *Aedes* transmitted arboviruses. *Gates Open Research* 2, 36.

Quinlan, M.M., Mumford, J.D., Knight, J.D. and Stonehouse, J.M. (2008) *Model Business Plan for a Sterile Insect Production Facility*. IAEA-MBP. International Atomic Energy Agency, Vienna, Austria.

Scott, T.W., Takken, W., Knols, B.G.J. and Boëte, C. (2002) The ecology of genetically modified mosquitoes. *Science* 298, 117–119.

Simmons, G., McKemey, A., Morrison, N., O'Connell, S., Tabshnik, B. *et al.* (2011) Field performance of a genetically engineered strain of pink bollworm. *PLoS ONE* 6, e24110.

Sinkins, S.P. and Gould, F. (2006) Gene drive systems for insect disease vectors. *Nature Reviews Genetics* 7, 427–435.

Sterner, R.T., Meltzer, M.I., Shwiff, S.A. and Slate, D. (2009) Tactics and economics of wildlife oral rabies vaccination, Canada and the United States. *Emerging Infectious Diseases* 15, 1176–1184.

Sun, H., Dickens, B.L., Richards, D., Ong, J., Rajarethinam, J. *et al* (2021) Spatio-temporal analysis of the main dengue vector populations in Singapore. *Parasites & Vectors* 14, 1–11.

Syed, R.A., Law, I.H. and Corley, R.H.V. (1982) Insect pollination of oil palm: introduction, establishment and pollinating efficiency of *Elaeidobius kamerunicus* in Malaysia. *Planter* 58, 547–561.

Undurraga, E.A., Halasa, Y.A. and Shepard, D.S. (2016) Economic analysis of genetically modified mosquito strategies. In: Adelman, Z. (ed.) *Genetic Control of Malaria and Dengue*. Elsevier, Oxford, UK, pp. 375–408.

Waltz, E. (2021) First genetically modified mosquitoes released in the United States. *Nature* 593 (7858), 175–176.

WHO and IAEA (2020) Guidance framework for testing the sterile insect technique as a vector control tool against *Aedes*-borne diseases. *World Health Organization, Geneva, Switzerland. Available at*: https://apps.who.int/iris/handle/10665/331679 (accessed 27 April 2022)

Zeddies, J., Schaab, R., Neuenschwander, P. and Herren, H. (2001) Economics of biological control of cassava mealybug in Africa. *Agricultural Economics* 24, 209–219.

27   The Cartagena Protocol on Biosafety and the Regulation of Transboundary Movement of Living Modified Organisms

Ricardo Pereira*

Cardiff University, Law & Politics School, Cardiff, Wales UK

27.1   Introduction

This chapter assesses the strengths and weaknesses of the present international legal framework involving the transboundary movement of genetically modified organisms. It also discusses the extent to which international law framework for the transboundary movement of living modified organisms (LMOs) is capable of dealing with the legal challenges surrounding the movement of transgenic insects.

The chapter starts by outlining the key tenets behind the Convention on Biological Diversity (1992) (adopted 5 June 1992, entered into force 29 December 1993) and its Protocol on the Transboundary Movement of Living Modified Organisms adopted in Cartagena in 2000 (hereafter, 'the Cartagena Protocol'). This is followed by an analysis of the specific provisions of the Cartagena Protocol, including the Supplementary Protocol on Liability and Redress agreed in the COP-10 in Nagoya–Kuala Lumpur in October 2010. The last section of the chapter discusses the implementation of the Protocol in the European Union in order to illustrate the challenges in the transposition and implementation of the Protocol.

27.2   Overview of the UN Convention on Biological Diversity

The 1992 Biological Diversity Convention (CBD) had been negotiated since 1988 and concluded at the Rio conference on Environment and Development ('the Earth Summit') in 1992, UNCED (UN Conference on Environment and Development)[1] with the main objectives of ensuring the conservation of biodiversity as well as the sustainable use of biological resources and access and benefit sharing of genetic resources (Article 1, CBD (1992)). It also regulates the handling and distribution of benefits of biotechnology.[2] This was the first attempt by the international community to adopt rules on biotechnology at the global level. The CBD

---

*Email: PereiraR1@cardiff.ac.uk

entered into force on 29 December 1993 and has presently virtually universal participation, with 196 parties (including the European Union), but with the notable absence of the USA.[3]

As is the case with many other multilateral environmental agreements, the CBD is a 'Framework Convention' laying down various guiding principles for implementation in national laws and policies, but its provisions are in general expressed as overall goals (using broad terms and vague language) rather than prescribing the precise legal obligations to be followed by the parties. This technique in international environmental law-making has the advantage of allowing for the maximum participation by states, who can at a later stage strengthen their legal obligations through the adoption of Protocols and amendments to the framework Treaty.[4] This allows for more specific and detailed requirements and standards to be decided once a framework for cooperation is in place and thus for the evolution of the regulatory regime.[5] Hence the CBD largely leaves it to individual states to determine how best to implement its legal provisions. This technique is unlike that deployed in other international agreements, such as the Convention on International Trade in Endangered Species of Fauna and Flora (CITES, 1973), which has specific trade restrictions and requirements for specific lists of species.

Although the CBD itself only deals with genetically modified organisms in very general terms,[6] some legal provisions of the CBD are particularly relevant to the question of deliberate release of genetically modified organisms. In relation to invasive species, the Convention calls on states to 'prevent the introduction of, control or eradicate those alien species which threaten ecosystems, habitats and species'.[7] Moreover, the parties are required 'as far as possible and appropriate' to introduce appropriate procedures to undertake environmental impact assessment (EIA) of proposed projects 'likely to have significant adverse impacts on biological diversity'.[8]

In decision 14/34, the Conference of the Parties (COP) under the CBD set out the process for developing a post-2020 global biodiversity framework to replace the Aichi 2010 Biodiversity Targets and established the Open-ended Working Group on the Post-2020 Global Biodiversity Framework to support this process. The Aichi Targets aimed at the implementation of the CBD Strategic Plan for Biodiversity (2011–2020) (CBD, 2010). The first draft of the post-2020 Global Biodiversity Framework for a new 10-year framework was adopted by the Working Group on the Post-2020 Framework on 23 August – 3 September 2020 (CBD, 2021). It is anticipated that COP-15, which was due to be held in Kunming in China in October 2021 and in April–May 2022 but had been subject to postponement, will provide a platform for adoption of the post-2020 global biodiversity framework.

27.3   Cartagena Protocol on Biosafety (2000/2003)

Although there are many benefits arising from biotechnology, for example for medical and pharmaceutical or for agricultural and nutritional purposes, there are also real risks that biotechnology can pose to biological diversity as well as to humans (Beyerlin and Marauhn, 2011).

The parties to the CBD had from the outset recognized the need for a protocol on genetically modified organisms (also called living modified organisms or LMOs).[9] After difficult negotiations, the Protocol was adopted on 29 January 2000 and entered into force on 11 September 2003.[10] The Protocol implements the obligations of the parties regarding biodiversity protection within the context of the transboundary movement of LMOs. The Protocol has attracted 173 ratifications to date (with the latest ratification by Sierra Leone on 15 June 2020) and therefore it is not binding on all parties to the CBD. Importantly, to date, the Cartagena Protocol on Biosafety has not been ratified by Canada, Argentina or the USA, who are major producers of genetically modified (GM) food and feedstocks. So, although all parties to the Protocol are parties to and therefore bound by the CBD, not all CBD parties are bound by the Protocol.[11]

The Protocol establishes specific risk assessment and risk management procedures governing the cross-boundary movements of LMOs with the view of ensuring an adequate level of protection for the transfer, handling and use of LMOs and thus to protect biological diversity from the potential environmental and health risks resulting from modern biotechnology.[12] The basis for the risk assessment procedure under the Biosafety Protocol is the precautionary principle.[13]

The scope of the Protocol is limited to 'the *transboundary movement*, transit, handling and use of all living modified organisms',[14] so it expressly rules out the application of the Protocol to merely internal situations, for example if the LMO is produced in one country and then released into that country's environment. One related contentious issue is whether the Protocol applies to transboundary movements from one party to a non-party. Under the Vienna Convention on the Law of Treaties (UN, 1980), a treaty or protocol cannot create rights and obligations for non-Parties without their consent. However, the Protocol does provide some degree of regulation in relation to transboundary movements of LMOs involving non-Parties.[15] According to the Protocol, such transboundary movements must be consistent with the objective of the Protocol and may be the subject of bilateral, regional and multilateral agreements between Parties and non-Parties.[16]

The Cartagena Protocol clearly applies to GM insects as per the definition of LMOs under the Protocol: 'any living organism that possesses a novel combination of genetic material obtained through the use modern biotechnology',[17] in particular those intended for release into the environment (e.g., seeds for cultivation or animal breeding stock)[18] or for use in food and feed, or for processing (e.g., maize, cotton and soy) (Arts. 7–10). The application of the Protocol to the deliberate release of LM mosquitoes is further evidenced by the work of the Sub-Working Group on LM mosquitoes to the Ad Hoc Technical Expert Group on 'Risk Assessment and Risk Management', in particular under its first guidance document on the release of LM mosquitoes (Marshall, 2011).

This guidance document, presented to the Fifth Meeting of the Conference Serving as the Meeting of the Parties to the Cartagena Protocol on Biosafety in October 2010, outlines a number of potential risks that must be considered prior to an open release of LM insects.[19] The applicability of the Cartagena Protocol to living modified mosquitoes was also confirmed by the conclusion of an Ad Hoc Technical Expert Groups (AHTEG) on Synthetic Biology formed by the Subsidiary Body on Scientific, Technical and Technological Advice (SBSTTA) that living organisms developed through synthetic biology are similar to LMOs as defined in the Cartagena Protocol.[20] The Protocol also regulates the *unintentional* movement of LMOs by establishing notification and emergency measures obligations when the party is aware of the occurrence of an unintentional movement which is likely to have 'significant adverse effects' on the conservation and sustainable use of biodiversity, taking into account risks to human health.[21] However, there is no definition under the Protocol of the term 'significant adverse effects', thus leaving a significant margin of discretion on state parties in how to implement the obligations under Article 17 of the Protocol.[22]

There was contention from the outset of the negotiations of the Protocol regarding the appropriate treatment of human health issues. Although Article 19(3) of the CBD makes no reference to human health, ultimately the Protocol negotiators reached a compromise and the Protocol recognizes throughout a number of its provisions that risks to human health are to be 'taken also into account.'[23] Although this falls short of a prohibition on transboundary movement of LMOs that can have an adverse impact on human health, it requires, at the very least, that human health issues are addressed in decision-making processes. Previous research has considered the possible risks of GM insect technology to environmental and human health (CBD, 2016; Lin, 2017; Schwindenhammer, 2020).

The implementation of the Protocol requires substantial human, financial and technical resources from both developing

and developed countries. In line with the principle of common but different responsibilities, the Convention makes implementation by developing countries dependent on the fulfilment of the financial aid obligations by developed countries.[24] In this vein, the Global Environmental Facility is entrusted with providing financial resources under the CBD and the Protocol for capacity building in the form of projects on national biosafety.

27.3.1   The Advanced Informed Agreement procedure

At the heart of the Protocol is the Advanced Informed Agreement (AIA) procedure, which aims to ensure that countries are provided with the information necessary to make informed decisions before agreeing to the import of LMOs into their territory.[25] The AIA procedure requires approval by the designated national authority before the import of LMOs for the intentional introduction into the environment of the importing party.[26] It requires that, before the first transboundary movement of an LMO, the party of import is notified of the proposed transboundary movement and is given an opportunity to decide whether or not the import shall be allowed and upon what conditions. This decision must be based upon a risk assessment (further discussed in section 27.3.2 below). According to the Protocol, failure to respond to initial notification does not imply consent to transboundary movement.[27]

The procedure is only to be followed if the LMOs are going to be *intentionally* introduced into the environment. A less stringent regime applies as regards the transboundary movements of LMOs-FFP (those intended for food, feed or processing): documentation accompanying such movements should clearly identify that they 'may contain' LMOs and that they are not intended for intentional introduction into the environment.[28] Given the scope of the present chapter, the focus of the analysis will be on the deliberate release of LMOs into the environment, rather than for use in food or feedstocks.

The AIA procedure is modelled loosely on existing mechanisms in international law for the transboundary movement of hazardous substances, for example the prior informed consent (PIC) procedures in the 1989 Basel Convention on the transboundary movement and disposal of hazardous wastes (UN, 1989) and the 1998 Rotterdam Convention on international trade in hazardous chemicals (UN, 1998; Mackenzie *et al.*, 2003). However, it is important to note that the Protocol allows for a significant degree of flexibility to Parties as to whether to apply the AIA procedure set out in the Protocol or instead use a different regulatory procedure available under national law, which must, none the less, be consistent with the Protocol.[29] The flexibility and discretion accorded to Parties under the Protocol means that the procedure to be followed by the exporting country and importing country in any given case may vary significantly depending on, for example, the regulation of the LMO in question under national law (ibid.). Moreover, the AIA procedure is not required under the Cartagena Protocol when an LMO is 'not likely to have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health'.[30] In this case, the importer only needs to notify a state of its intent to introduce into the market an LMO through the Biosafety Clearinghouse (Telesetsky, 2011). This could lead to a loophole in the system when the national risk assessment procedure in the importing state (or exceptionally in the exporting state (Box 27.1)) is inappropriately carried out and fails to identify risks to human health and the environment.

Further, the Protocol sets out the details of the AIA procedure, establishing for example the length of time for the Party of import to make a decision whether to allow or to prohibit the import of the LMO[31] and on which basis the decision must be made.[32] It has been suggested that the flexibility accorded to Parties under the Protocol, and the terms of the AIA provisions of the Protocol, could give rise to some ambiguity and uncertainties in the application of the Protocol in practice (Mackenzie *et al.*, 2003).

Box 27.1. The Advanced Informed Assessment (AIA) and the Transboundary Movement of Transgenic Mosquitoes for Contained Use

---

Drawing from the experiences of countries that have recently introduced GM mosquitoes, such as the Cayman Islands[33] and Malaysia,[34] John Marshall (2011) argued that:

> One weakness [of the Protocol], highlighted by recent exports of LM mosquito eggs from the United Kingdom, is that the Advance Informed Agreement procedure does not apply to LM mosquitoes being considered for release following laboratory studies and/or cage trials in the receiving country. This means that, under the most likely release scenario for any LM mosquito, the exporting country is not required to perform and finance a risk assessment.

Indeed, Article 6(2) of the Protocol states that 'the provisions of this Protocol with respect to the advance informed agreement procedure shall not apply to the transboundary movement of living modified organisms destined for contained use undertaken in accordance with the standards of the Party of import'.

This can be a problem, since 'in almost all cases LM mosquitoes are first exported for careful analysis in laboratory studies and cage trials in the receiving country, and the importing country is not entitled to request the exporting country to perform a risk assessment under these circumstances' (Marshall, 2011). This means that once LM mosquitoes have been received by the importing country, the country is free to release them into the environment in accordance with their own national regulations (ibid.).

Although it could be argued that the inapplicability of the AIA procedure to LM organisms imported for contained use is in principle justifiable by the fact that the AIA procedure requires an evaluation of the receiving environment as part of the risk assessment (Annex III of the Protocol), Marshalll (2011) suggested that 'for strains sent for initial laboratory analysis or cage trials, release sites may not be identified and open releases may not even by planned'.

During the negotiation of the Protocol, there was debate as to whether the AIA procedure should apply to every transboundary movement of an LMO into a Party or only to the first transboundary movement of a specific LMO into a Party of import. Article 7(1) of the Protocol clarifies this question by providing that AIA shall only apply to the *first* intentional transboundary movement of LMOs into the environment of the Party of import (Mackenzie *et al.*, 2003). A plain reading of Article 7(1) could lead one to interpret that AIA procedure applies where a particular LMO is to be introduced into the Party of import *for the first time from any other Party to the Protocol*, and that AIA does not apply automatically *each time the same LMO is subsequently imported from other Parties*. This would mean that if an import permit is issued for the first transboundary movement of the LMO in question, the AIA procedure would not be required for subsequent imports. Yet such an interpretation might give rise to some difficulties for the Party of import because if it approves the first import of a specific LMO from another

Party, then for subsequent imports it might not be able to assess whether the content of what is being imported is in fact the 'same' LMO that has already been approved under the AIA procedure (ibid.). This could be a problem in particular in the absence of a unique identification mechanism for LMOs across countries.[35]

27.3.2 Risk assessment and public participation

As discussed above, the AIA procedure requires that a risk assessment be carried out prior to the transboundary movement of LMOs. Annex III of the Protocol sets out the requirement for risk assessment prior to the transboundary movement (see Hayes and Quinlan, Chapter 28, this volume for risk assessment of GM insects). The Protocol establishes that the importing party bears the responsibility to ensure that a risk assessment is carried out.[36]

According to Article 15, risk assessments undertaken pursuant to the Protocol

shall be carried out in a *scientifically sound manner*, in accordance with Annex III and taking into account recognized risk assessment techniques. Such risk assessments shall be based, at a minimum, on information provided in accordance with Article 8 and other available scientific evidence in order to identify and evaluate the possible adverse effects of living modified organisms on the conservation and sustainable use of biological diversity, taking also into account risks to human health. In this vein, the Protocol upholds the precautionary principle by stating that 'lack of scientific knowledge or scientific consensus should not necessarily be interpreted as indicating a particular level of risk, and absence of risk, or an acceptable risk'.[37] This does not imply that members can invoke the precautionary principle without risk assessment, but merely that the assessment can be made on the basis of factors that would not otherwise fulfil the stringencies of the assessment standard.[38]

At a minimum, risk assessment for LMOs subject to the AIA procedure is to be based on information provided in accordance with Article 8 of the Protocol, the information specified in Annex I and other available scientific evidence. Relevant scientific evidence to be taken into account includes scientific data (including statistical data, if available), scientific theories, models and other sources of scientific knowledge, that assist in the identification of possible adverse effects, and evaluation of the probability of adverse effects occurring, and of their consequences (Mackenzie *et al.*, 2003). In addition, risk assessments of LMOs under Article 15 are to take into account recognized risk assessment techniques. The Protocol does not specify what constitute recognized risk assessment techniques, but they may be assumed to include those techniques that are currently applied at national, regional or international level.[39]

The COP-13 of the CBD held in Cancun, Mexico, in 2016 noted that the general principles and methodologies for risk assessment under the Cartagena Protocol and existing biosafety frameworks provide 'a good basis' for risk assessment of living organisms developed through synthetic biology, but that

such methodologies might need to be updated and adapted (WHO, 2021 Guidance Framework p. 144; CBD, 2017, para 1). Moreover, the AHTEG noted that existing risk assessment considerations 'might not be sufficient or adequate to assess and evaluate the risks that might arise from organisms containing engineered gene drives due to limited experience and the complexity of the potential impacts on the environment' (CBD, 2017, para 44). In addition, the 2018 COP-14 decision called for precaution regarding uncertainties for engineered gene drive and additional guidance for risk assessment.[40]

It has been suggested that the risks associated with the release of GM insects vary regarding the type of GM insect strategy, targeted species and geographical context (Swindenhammer, 2020). Significantly, the WHO Guidance Framework for Testing of Genetically Modified Mosquitoes (first published in 2014, and revised and updated in 2021) provides guidelines on testing and has set out standards for assessing the safety and efficacy of GM insects (WHO, 2014; WHO, 2021). However, unlike the Cartagena Protocol, the WHO guidelines are non-binding.

The public participation provisions of the Cartagena Protocol require the State Party to ensure that the public is actively consulted on decisions relating to LMOs and biosafety 'in accordance with their respective national laws and regulations' (see Thizy *et al.*, Chapter 24, this volume for a discussion of public participation and GM insects).[41] LMOs were expressly excluded from the provisions on public participation of the UNECE Aarhus Convention on Access to Information, Public Participation in Decision-Making and Access to Justice in Environmental Matters, which only requires Parties to apply the Convention's public participation provisions 'to the extent feasible and appropriate' to decisions relating to deliberate release of GMOs (Article 6(11))[42]. However, in May 2005 there was agreement on an amendment to the Aarhus Convention[43] requiring parties to inform and consult the public on decision making regarding the release of genetically modified organisms (GMOs), with the caveat that this obligation

was subject to protection of commercially confidential information (Birnie *et al.*, 2009). Yet this amendment must be ratified by three-quarters of the states which were Parties to the Convention at the time of the adoption of the amendment before it enters into force.[44] Moreover, State Parties to the Aarhus Convention are guided to apply the Guidelines on Access to Information, Public Participation and Access to Justice with respect to Genetically Modified Organisms (the 'Lucca Guidelines')[45] which are, however, non-binding. In this regard, in 2016 the House of Lords Science and Technology Committee noted that appropriate public engagement strategies will have a critical role to play in the development and progression of GM insect technologies and has recommended that 'engagement with the public, both in the UK and overseas, particularly in countries where insect-borne disease is rife, will be required'. (House of Lords, 2016, para. 170).

27.3.3   Liability and compliance

One of the main weaknesses of the CBD is that it does not establish a compensation scheme for environmental damage. Article 14 merely calls on parties to 'examine … the issue of liability and redress, including restoration and compensation, for damage to biodiversity except where such liability is purely an internal matter'. Therefore, there is no compensation mechanism under the CBD which could effectively deal with the claims of liability and redress arising from environmental damage.

The CBD recognizes a number of mechanisms for settlement of disputes available to the parties.[46] In decision BS-1/7, the COP-MOP (Conference of the Parties/Meeting of the Parties) established a standing Compliance Committee (Mackenzie, 2004); however, there was 'failure to agree on further measures to respond to repeated non-compliance'. Moreover, by the time it was set up, the rules on liability had not been resolved and 'for some issues, such as liability and identification requirements, the

COP-MOP has already established Ad Hoc groups to undertake further negotiations' (Mackenzie, 2004). McGraw (2002) suggested that 'since many of the most contentious issues were left unresolved at the time of the CBD's adoption, the post-agreement negotiations have proven particularly challenging'. The non-compliance issues can therefore be taken before the Compliance Committee, but the consequences are still unclear in the event that a party is found to be in breach of the Protocol. However, Decision I/7 provides for the kind of measures that can be taken both by the Compliance Committee and by the COP-MOP, which includes requesting a party to develop an action plan towards compliance as well as other financial, technical or other assistance to the parties (Mackenzie *et al.*, 2003). The enforcement measures available to the committee fall short of stronger binding measures with a punitive character, as was the case with the non-compliance mechanism under the Kyoto Protocol to the UNFCCC, which included punitive sanctions for states' non-compliance with emissions reductions targets and other obligations under the Protocol.[47]

There are only two avenues for complaints to be brought before the Cartagena Compliance Committee. Namely, the Committee shall receive, through the Secretariat, submissions relating to compliance from: (a) any Party with respect to itself; and (b) any Party, which is affected or likely to be affected, with respect to another Party. To date, however, the Compliance Committee has not yet received any submission from a party with respect to itself or any party-to-party submission.[48] This can be explained because national governments will be reluctant to bring a case in respect to themselves, and they may not wish to take procedures forward in case they themselves are found to be in violation with parts of the Protocol.[49] Hence there are no effective non-compliance mechanisms available for the parties to raise their claims under the Convention or Protocol, at present.[50] It has been suggested that the CBD's enforcement provisions are 'vague and voluntaristic (at best)' and 'confusing and contradictory (at worst)' (McGraw, 2002). Moreover, the compliance mechanism suffers

---

Box 27.2. Overview of the Cartagena Protocol Non-Compliance Procedure.

Arguably, one of the most significant developments in the field of international environmental law has been the emergence of non-compliance procedures under various Multilateral Environmental Agreements (MEAs), with a number of treaties establishing subsidiary bodies to deal with disputes over non-compliance (Sands and Peel, 2012). The first non-compliance procedure in an MEA was established under the 1987 Montreal Protocol on phasing out of ozone-depleting substances, under which any party may submit a complaint to the Implementation Committee regarding a violation by another party of the Protocol (Pereira, 2012). The Committee may undertake information gathering in the territory of the party concerned (at the invitation of the party) and has the power to suspend specific rights and privileges under the Protocol (ibid.; Sands and Peel, 2012).

The Cartagena Compliance Committee may take a number of measures with a view to promoting compliance and addressing cases of non-compliance. These include the following.

- Providing **advice or assistance** to the party concerned.
- Making recommendations to the COP-MOP regarding **the provision of financial and technical assistance**, technology transfer, training and other capacity-building measures.
- Requesting or assisting the party concerned to develop a **compliance action plan** regarding the achievement of compliance with the Protocol within a timeframe to be agreed upon between the Committee and the party.

Moreover, the COP-MOP may, upon the recommendations of the Compliance Committee, decide upon one or more of the following measures.

- Provide financial and technical assistance.
- Issue a caution to the concerned Party.
- Request the Executive Secretary to publish cases of non-compliance in the Biosafety Clearing-House.
- In cases of repeated non-compliance, take such measures as may be decided by the COP-MOP at its third meeting.

See further on the Cartagena non-compliance procedure: http://www.cbd.int/biosafety/issues/compliance.shtml (accessed 25 August 2021).

---

from some inherent weaknesses that may ultimately put its credibility at risk, in particular that no financial resources are available to the Compliance Committee or the COP-MOP itself for implementing recommendations to provide assistance to non-compliant parties (Koester, 2013).

Further limitations of the Protocol's non-compliance procedure were evident in the Sixth Meeting of the Committee, in which it considered whether it had a mandate to receive and consider a submission made by a non-governmental organization (NGO) alleging non-compliance of a party with its obligations under the Protocol. The Committee concluded that it has no mandate to consider the submission, because section IV of the compliance procedures adopted in the annex to decision BS-I/7 permits only a party to trigger the procedures with respect to itself or with respect to another party. [51, 52]

27.3.4 The Nagoya–Kuala Lumpur Supplementary Protocol on Liability and Redress to the Cartagena Protocol on Biosafety

The Cartagena Protocol calls on states to adopt provisions on liability, redress and compliance.[53] This provision was negotiated in particular under the insistence from many developing countries who argued that the transboundary movement of LMOs should only be permitted if the allocation of costs of any adverse effects was regulated through a civil liability regime (Lefeber, 2012; Nijar 2012). In COP-MOP 4 (2008), the parties agreed to draft legally binding rules and procedures for liability and redress for potential damage arising from transboundary movement of LMOs, which were to be put forward for consideration in the following COP in 2010.

The Nagoya–Kuala Lumpur 'Supplementary Protocol',[54] adopted in COP-10/MOP-5,

provides international rules and procedures on liability and redress for damage to biodiversity resulting from LMOs. It entered into force on 5 March 2018 following the receipt of the 40th instrument of ratification. At the time of writing there are 49 State Parties to the 'Supplementary Protocol', with the most recent ratification by Austria in May 2021.[55]

The Supplementary Protocol establishes a legal framework that allows countries importing GMOs to make the producer in the exporting country liable for any possible damage caused by the imported GMOs and which may have an impact 'on conservation and sustainable use of biological diversity, taking into account risks to human health'.[56] The Supplementary Protocol applies to damage resulting from LMOs which find their origin in a transboundary movement (intentional or non-intentional).[57] Hence the Protocol has a broader scope than the Cartagena Protocol itself, in that it covers not only damage caused by intentional transboundary movement, but goes further when regulating non-intentional transboundary movement of GMOs (Article 3(3)).[58] As has been discussed above, this is in line with the decision-making processes often used in international environmental law, which allows the parties to strengthen their legal obligations under a framework treaty through the adoption of subsequent protocols.

The Supplementary Protocol applies to damage[59] that occurred in areas within the limits of the national jurisdiction of Parties.[60] Specific provisions allow Parties to retain the right to provide, in their domestic law, for financial security.[61] However, unlike the international civil liability regimes for oil pollution or nuclear accidents, there is no mandatory requirement that the operator is covered by insurance under the Supplementary Protocol.[62] The rules under the Protocol are predominantly procedural in nature (Lefeber, 2012). According to Article 5(2), the competent authority shall: (a) identify the operator which has caused the damage; (b) evaluate the damage; and (c) determine which response measures should be taken by the operator. Another way to limit the application of the liability regime is, according to Article 6(1), for the parties to provide in their domestic law for exceptions from liability under the following circumstances: (a) Act of God or *force majeure*; and (b) act of war or civil unrest. Moreover, significant leeway for implementation is given to parties under Article 6(2), which allows parties to provide, in their domestic law, *for any other exemptions or mitigations as they may deem fit*. Furthermore, according to Article 3(7) of the Supplementary Protocol, domestic law implementing the Supplementary Protocol shall also apply to damage resulting from transboundary movements of LMOs from non-parties.[63] So the Supplementary Protocol goes further than the Cartagena Protocol itself, which, as noted above, only provides for limited regulation of transboundary movements of LMOs involving non-parties.

Although the scope of the Cartagena Protocol is specifically limited to the first transboundary movement of each particular LMO[64] into the importing country, the Supplementary Protocol on liability and redress apparently does not contain a similar limitation to damage attributable to the 'first international introduction'. Hence the 'first international movement' limitation applies only to the Cartagena Protocol's mechanisms (for example, as regards the AIA procedure and risk assessment requirements) but not to the assignment of damage arising from the transboundary movement regulated under the Nagoya–Kuala Lumpur Supplementary Protocol (Tsioumani, 2010).[65]

Although the Supplementary Protocol allows for claims to be made by the importing state against the exporter, it imposes no significant obligation on the exporting state to impose liability on entities under their jurisdiction for LMO-related damage to other states. Hence the enforcement of the obligations under the Supplementary Protocol is dependent upon action being taken by the importing state.

Overall, the Nagoya–Kuala Lumpur Supplementary Protocol, like its founding Cartagena Protocol, fails to introduce mandatory language in most liability provisions, and includes a number of provisions that appear to enable parties to controvert and effectively invalidate all aspects of the

Supplementary Protocol that may apply to them (Tsioumani, 2010). This vague and ambiguous language applied, which is aimed at ensuring flexibility in implementation, is evidenced for example in Article 6(2) of the Supplementary Protocol, under which parties are specifically authorized to 'provide, in their domestic law, for any other exceptions or mitigations as they deem fit' and 'to set any temporal limits they choose on actions alleging LMO-based liability, and/or to cap the maximum claim that may be recovered in such cases'. Indeed, the Protocol reserves its mandatory provisions for the country where the injury occurs, rather than the country that has jurisdiction over the operator and is better able to institute proceedings against the defendant (Tsioumani, 2010). In this context, in an unlikely provision under the protocol deploying mandatory language, the Supplementary Protocol states that in the event of LMO-related damage, the operators involved are required to 'immediately inform the competent authority', 'evaluate the damage' and 'take appropriate response' measures with regards to such damage (Article 5(1)). A similar obligation is placed on the competent authority in the country where damage occurred. It should be noted that another limitation of the Nagoya–Kuala Lumpur Supplementary Protocol is that it could not apply to purely internal situations, as Article 14(2) of the CBD states that 'the Conference of the Parties [COP] shall examine, on the basis of studies to be carried out, the issue of liability and redress, including restoration and compensation, for damage to biological diversity, *except where such liability is a purely internal matter*' [emphasis added].

The establishment of liability is obviously dependent on the identification of one or more individuals or entities who are the operators.[66] National laws regarding liability and remedies will govern any liability action or claim for redress. Where the operator does not take appropriate measures, the protocol notes that the country may take those measures instead, recovering its costs from the operator. However, as is the case with other civil liability regimes established under other international environmental

agreements,[67] the liability of the operator is without prejudice of the objective liability of the state under international law for transboundary environmental harm. Moreover, it is important to note however that the supplementary protocol only establishes minimum standards as regards the *civil* liability of the operator for the damage, so it is without prejudice of any criminal liabilities established under national law (unlike civil liabilities, criminal liabilities are non-insurable, and may include imprisonment and other more coercive penalties).[68]

Despite the limitations and weaknesses of the Nagoya–Kuala Lumpur Supplementary Protocol, the private sector developed a proposal for self-regulation that eventually evolved into a contractual mechanism for liability and redress in response in the event of damage to biological diversity caused by the release of a living modified organism, known as 'Compact'. The membership of Compact consists of six companies with large market shares in the agricultural biotechnology market, but it is in principle open to other entities that meet the conditions of membership (Lefeber, 2012).

27.3.5   The implementation of the Cartagena Protocol – the case of the European Union

The EU transposed its obligations under the Cartagena Protocol through Regulation (EC) No. 1946/2003 on Transboundary Movements of Genetically Modified Organisms, which governs the intentional and unintentional movements of GMOs between Member States and exports of GMOs to third countries by requiring the prior and informed consent of the importing state. Yet the main regulation in the EU governing the deliberate release of GMOs is Directive 2001/18/EC on the Deliberate Release into the Environment of Genetically Modified Organisms ('The Deliberate Release Directive').[69] It applies to both the experimental release of GMOs into the environment, that is, the introduction of GMOs into the environment for experimental purposes, for example for

field testing (mainly covered by Part B thereof), and the placing on the market of GMOs (products containing or consisting of GMOs), for example for cultivation, import or processing into industrial products (mainly covered by Part C thereof).[70] GM insects, which might be produced in academic settings and implemented by for example Ministry of Health and released directly into the environment without commercialization, are hence covered by Part B of the Deliberate Release Directive without the requirement of EU approval (House of Lords, 2015–2016).[71]

The Directive establishes a centralized EU authorization procedure for placing GMOs on the market as or in products, where the intended use of the product involves the deliberate release of the organism(s) into the environment.[72] The Directive contains principles for environmental risk assessment and the requirement for companies intending to market a GMO to obtain written authorization. It requires the EU Member States to ensure, in accordance with the precautionary principle,[73] that all appropriate measures are taken to avoid adverse effects on human health and the environment as a result from the deliberate release or the placing in the market of GMOs.[74,75] The European Food Safety Authority (EFSA) performs the scientific risk assessment at the EU level and serves as the scientific advisory body that informs the European Commission.

The EU GMOs regulatory framework is 'without prejudice of additional requirements laid down by specific Community legislation'. In particular, there are additional EU-wide liability regimes set out in secondary legislation. In this regard, the Environmental Liability Directive[76] applies to the placing of GMOs into the EU market, requiring the Member States to establish a specific civil liability regime providing remedial action for environmental damage, which is to be overseen by competent national authorities. Moreover, the Environmental Crime Directive (ECD)[77] requires the Member States to establish criminal offences in connection with serious violations of the Deliberate Release Directive (Pereira, 2015),[78] although it should be noted that the 'recast'

Environmental Crime Directive proposed by the European Commission in December 2021 to replace the ECD does not include a specific criminal offence relating to the transboundary movement of GMOs or deliberate release of GMOs into the environment (ECD, 2021).

As regards the procedure for the release of GMOs under the Deliberate Release Directive, an environmental risk assessment is required, in accordance with Annex II, before a notification is submitted, taking into account the impact on the environment according to the nature of the organism introduced and the receiving environment (Lee, 2008a). The aim of the assessment is to identify if there is a need for risk management and, if so, the most appropriate method to be used.[79]

Before a GMO or a combination of GMOs as or in products is placed on the market, a notification shall be submitted to the competent authority of the Member State where such a GMO is to be placed on the market for the first time. The notification must contain a range of information, including the environmental risk assessment carried out by the applicant, and a plan for monitoring the GMO following its release into the environment.[80] No deliberate release of GMOs should be carried out following the notification unless the written consent of the competent authority – designed by the Member States themselves – is granted.[81] The national competent authorities must be satisfied that the release will be safe for human health and the environment.[82] The competent authority examines the notification for compliance with the Directive and drafts an 'assessment report' indicating whether the GMO should (and under which conditions) or should not be placed on the market.[83] The assessment of risk is done on a case-by-case basis.[84]

In addition, Member States must take measures to ensure traceability, in line with the requirements laid down in Annex IV, at all stages of the placing on the market of GMOs that have been authorized (Lee, 2008b). Hence, following the placing a GMO on the market as or in a product, the notifier shall ensure that monitoring and reporting on it are carried out in accordance with the

conditions specified in the consent, and noting that the consent is time limited and must be renewed.

The EU is one of the largest importers for GMO-derived food and fuel, including soybean and soy meal.[85]

The 2001 Deliberate Release Directive was complemented by Regulation 1829/2003 on Genetically Modified Food and Feed (the 'Food and Feed Regulation'), which applies an obligation to seek authorization for any GMO destined for use in food or feed. According to the Regulation, no GMO food may be released into the EU market without the relevant conditions for authorization.[86] The Regulation has been a cause of major contention among certain EU trading partners, especially as regards the extent to which the regulation will apply to food and feed produced from or merely containing ingredients produced from GMOs.[87,88,89] This different regulatory framework under the 2003 Regulation applies depending on whether GMO is used in food or (animal) feed (but not a non-food GMO, such as GM insects), although the two pieces of legislation overlap. GMOs that have no food or feed use, as in the case of GM insects, are authorized under the Deliberate Release Directive alone (see Beech *et al.*, Chapter 25, this volume for a discussion of regulation of GM insects).[90]

Those regulatory requirements, with the exception of the EU's implementation of the Cartagena Protocol's provisions on the transboundary movement of GMOs, only apply to trials and commercial releases within the EU. Yet the main uses of GM insect technologies, particularly for public health purposes, will occur outside the EU. To date, there were only attempts to seek regulatory approval for GM insect technology in one EU Member State (Spain), but those attempts were unsuccessful.[91]

27.4 Conclusions

Despite the weaknesses of the Cartagena Protocol, which includes the use of vague and non-mandatory language, the Protocol has enabled countries to establish a relatively reliable system of authorization governed by national authorities before the transboundary movement of LMOs. Hence, based on advanced informed consent and risk assessment procedures, the Protocol succeeds in creating more transparency and security through establishing an international framework for governance of the transboundary movement of LMOs. Yet the effectiveness of the Protocol depends on its implementation by the national governance structures that are in place, and although the Protocol sets some important minimum handling, transport, packaging and identification requirements (see Article 18 of the Protocol), ultimately it will be for the importing countries to establish the conditions for import of the GMO in question.[92]

The application of the Protocol to the transboundary movement of LM mosquitoes, including the reforms relating to liability and redress introduced by the Nagoya–Kuala Lumpur Protocol, could have a significant impact on the activities of operators involved in the import and export of LM mosquitoes. In particular, previous research focusing specifically on the applicability of the Protocol to GM mosquitoes has suggested that there are weaknesses in practice in the application of the Protocol to GM mosquitoes, in particular the fact that it fails to require the AIA procedure in the case of GM mosquitoes destined for contained use (Marshall, 2011; Lin, 2017). This failure of the Protocol to adequately address issues relating to the transboundary movement of GM mosquitoes is not unconnected to the fact that the Protocol was written taking into account primarily LM crops (Marshall, 2011), although the Protocol does in fact cover 'organisms' more broadly.

Beyond the key issues addressed in this chapter, it is also clear that the trade-restrictive measures under the Protocol may conflict with World Trade Organization (WTO) law.[93] Thus, as discussed in the context of the EU, WTO law could hinder the ability of countries to forestall the importation of LMOs based on environmental or health grounds.[94] Yet a powerful tool remains on the side of importing countries, in that they

are allowed under the Protocol to ban, based on the precautionary principle, imports of GMOs because of lack of scientific certainty regarding the risks to the environment or human health, provided that the appropriate risk assessment procedures have been followed.[95] Importantly, as the USA is not a party to the Protocol and is a major exporter of GM products, there are concerns by civil society organizations that the USA may exercise its rights under the WTO to 'thwart any trade restrictive measures applied against it pursuant to those [multilateral environmental agreements] that it has chosen not to support' (Eckersley, 2004, p. 38). Therefore, unsurprisingly considering the contentious issues at stake, the global regulation and policies relating to the transnational cooperation and trade in GMOs are still imperfect and need to be reformed in order to strike the appropriate balance between the interests of importing countries (chiefly concerning environmental protection and human health) and the aim of GMO-producing countries to advance the interests of free trade in GM products and organisms.[96]

Notes

1. In addition to the CBD, another legally binding treaty was adopted in the Rio conference (the UN Framework Convention on Climate Change), and three non-binding documents, including the Rio Declaration on Environment and Development and the 'Forest Principles'.
2. See Article 19 thereof. It includes specific provisions on participation in biotechnological research (Art. 19.1), access to and distribution of the benefits of biotechnology (Art. 19.2), and access to and transfer of technology including biotechnology (Art. 16).
3. The USA initially refused to sign it for threatening to delay development of biotechnology and for not sufficiently protecting ideas. President Clinton signed the Convention but the US Congress has not ratified it.
4. These new obligations will in general only bind the parties that have ratified the Protocol, or voted for the amendment to a Treaty.
5. This is the case of the Convention on Biological Diversity (1992), the Cartagena Protocol (2000) and the Supplementary Nagoya Protocols on Access and Benefit Sharing (2010) and Liability and Redress (2010). Other examples in international law-making practice include the Climate Change Framework Convention (1992) and its Kyoto Protocol (1997); and the Vienna Convention on the Phasing Out of CFC Substances (1985) and its Montreal Protocol (1987).
6. Article 8(g) and Article 19(3).
7. Article 8(h).
8. Article 14. The obligation for states to undertake environmental impact assessment in transboundary situations has been recognized by the International Court of Justice in the Pulp Mills case (2010) regarding a dispute between Argentina and Uruguay relating to environmental management in the Uruguay river. See: Pulp Mills on the River Uruguay (Argentina v. Uruguay), Provisional Measures, Order of 13 July 2006, I.C.J. Reports 2006, p. 113, available at http://www.icj-cij.org/docket/files/135/11235.pdf.
9. See Article 19(3) CBD.
10. The first meeting of the Parties to the Protocol (MOP) was held 23 to 27 February 2004 in Kuala Lumpur, Malaysia.
11. In general, one of the requirements in international law before a state can become a party to a Protocol is that it first accedes or ratifies the 'Framework Treaty'.
12. Article 1.
13. Article 10(6), Cartagena Protocol. The principle is also enshrined in the Rio Declaration on Environment and Development (Principle 15). The precautionary principle is used to advocate that lack of scientific certainty is not an excuse for inaction against an environmental threat. It is suggested that where there are threats of serious or irreversible damage, lack of full scientific certainty should not be used as a reason for postponing such measures (see, for example, Article 3 of the UN Framework Convention on Climate Change).
14. Article 3(g) Cartagena. See also the UNIDO Voluntary Code of Conduct for the Release of Organisms into the Environment (1992).
15. See especially Article 24 of the Cartagena Protocol.

[16.] In accordance with Article 24.

[17.] Article 3(g).

[18.] Article 11.

[19.] However, issues relating to self-propagating living modified mosquitoes capable of spreading trans-genes beyond their release site are inadequately addressed and should be resolved in future guidance documents (ibid.).

[20.] See further at https://www.cbd.int/decisions/cop/12/28 (accessed 23 August 2021). This conclusion was 'taken note' by the Conference of the Parties (COP) to the CBD in Decision XIII/17. See also Report of the Ad Hoc Technical Group on Risk Assessment, 15 April 2020, available at https://www.cbd.int/https://www.cbd.int/doc/c/a763/e248/4fa326e03e3c126b9615e95d/cp-ra-ahteg-2020-01-05-en.pdf

[21.] Article 17 of the Protocol requires parties to notify affected or potentially affected states, the Biosafety Clearing House and relevant international organizations in such situations.

[22.] Yet there is a general duty in customary international law that states must cooperate to resolve common environmental problems in particular when activities in a state territory can cause environmental damage in another state or in areas beyond national jurisdiction (see e.g. Principle 2 of the Rio Declaration on Environment and Development). This calls for states 'to notify' and 'consult with' other affected states before the start of a project with transboundary implications (see e.g. ICJ, Pulp Mills case, Argentina v. Uruguay, 2010, above Note 8).

[23.] See e.g. Article 4: '[t]his Protocol shall apply to … [Living Modified Organisms, LMOs] that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health [emphasis added].

[24.] Article 20(4) CBD states that the extent to which developing country parties will effectively implement their commitments under the Convention will depend upon the effective implementation by developed country parties of their commitments under the Convention related to financial resources and technology transfer.

[25.] Articles 8–10 and 12, Cartagena Protocol.

[26.] Article 7(1), ibid.

[27.] Article 9(4), ibid.

[28.] Article 18(2)(a), ibid.

[29.] See Article 9, ibid.

[30.] Article 7(4).

[31.] Articles 9 and 10.

[32.] Articles 10 and 15; Annex III.

[33.] See Katherine Nightingale, 'GM Mosquito Wild Release Takes Campaigners by Surprise', SciDev Net (11 November 2010), reporting that Cayman Island released GM mosquitoes in 2009. Available at: http://www.scidev.net/en/news/gm-mosquito-wild-release-takes-campaigners-by-surprise.html (accessed 15 February 2013).

[34.] See Shiow Chin Tan, 'Malaysia to Release GM Mosquitoes into the Wild', SciDev Net (2 November 2010) (reporting Malaysia's intent to release GM male mosquitoes into their environment to combat dengue fever). Available at: http://www.scidev.net/en/nealaysiasia-to-release-gm-mosquitoes-into-the-wild.html (accessed 15 February 2013).

[35.] At its fifth meeting, the Conference of the Parties serving as the Meeting of the Parties to the Protocol (COP-MOP) acknowledged the importance of the detection and identification of LMOs by including specific outcomes in the Strategic Plan for the Cartagena Protocol on Biosafety to be achieved by 2020: See also decision BS-V/9 of the COP-MOP. See further: https://bch.cbd.int/protocol/cpb_detection/toolsandguidance.shtml

[36.] Article 15 states that 'the Party of import shall ensure that risk assessments are carried out ... It may require the exporter to carry out the risk assessment'. On this question see further: Hill et al. (2004).

[37.] The precautionary principle is elaborated in Principle 15 of the Rio Declaration on Environment and Development (1992). The Seabed Chamber of the International Tribunal on the Law of the Sea opined in February 2011 that the precautionary principle may have reached the status of customary international law. Case no. 17 Responsibilities and obligations of States sponsoring persons and entities with respect to activities in the Area (Request for Advisory Opinion submitted to the Seabed Disputes Chamber) (paras 131 and 135).

[38.] Under the WTO system the principle is no less problematic. While Article 5.1 of the Sanitary and Phytosanitary (SPS) agreement requires that measures by members be based on risk assessment, this shall most certainly not include cases of theoretical uncertainty. However, Article 5(7) literally embodies more

explicitly the precautionary principle, by permitting members to adopt provisional SPS measures where relevant scientific evidence is insufficient, provided that it subsequently seeks more objective assessment of the risk (to human health or the environment) within a reasonable period of time. In the EU–USA dispute over GMOs, discussed below, SPS provisions have been evoked to oppose the framework that governs the authorization of GMOs in the EU.

39. Examples of such techniques would include the UNEP International Technical Guidelines on Biosafety and the OECD's work on risk assessment. In this vein, in March 2006 Curitiba, Brazil, the Parties to the Protocol gathered for their third meeting to further advance the objective of the treaty; in particular, the Convention's governing body was asked to review the need for additional guidance on risk assessment and risk management and the need for establishing further subsidiary bodies under the Protocol to provide advice on scientific and technical matters.

40. The AHTEG was convened and reported on in April 2020. See CBD/CP/RA/AHTEG/ 15 April 2020, available at: cbd.int/doc/c/a763/e248/4fa326e03e3c126b9615e95d/cp-ra-ahteg-2020-01-05-en.pdf (accessed 28 April 2020).

41. Article 23(2), Cartagena Protocol.

42. The United Nations Economic Commission for Europe (UNECE) Aarhus Convention on Access to Environmental Information, Access to Justice and Participation in Decision-Making, adoption 25 June 1998; entry into force 30 October 2001.

43. In Decision II/1 at its second session (Almaty, Kazakhstan, 25–27 May 2005), the Meeting of the Parties adopted an amendment to the Convention, available at: https://unece.org/DAM/env/documents/2005/pp/ece/ece.mp.pp.2005.2.add.2.e.pdf. See also Article 6 (11) of the Aarhus Convention. See further, UNECE, 'The Aarhus Convention: An Implementation Guide' (Second edition, 2014), available at: https://unece.org/DAM/env/pp/Publications/Aarhus_Implementation_Guide_interactive_eng.pdf

44. 27 State Parties (as of May 2005) must ratify the GMO amendment. At the time of writing, the amendment has been ratified by 26 States which were parties to the Convention at the time of adoption of the amendment.

45. Adopted by the Meeting of the Parties at its first session in Lucca, Italy, on 21–23 October 2002. Available at: https://unece.org/DAM/env/pp/documents/gmoguidelinesenglish.pdf

46. Article 27 of the CBD addresses the settlement of disputes. It establishes a graduated response to resolving disputes, first requiring the parties concerned to seek a solution by negotiation, then allowing a third party to mediate or provide good offices if negotiation has not been successful. Paragraph 3 allows the Parties to agree to submit disputes to arbitration in accordance with Part 1 of Annex II to the Convention and/or to the jurisdiction of the International Court of Justice. If the parties to a dispute have not submitted to either of these procedures, the dispute is to be submitted to conciliation in accordance with Part 2 of Annex II unless the parties agree otherwise. None of these dispute settlement mechanisms have been used to date, perhaps because the Convention leaves much of the specifics of its implementation to be determined by each country in light of its own domestic circumstances.

47. On the Paris Agreement's non-compliance procedure, see further C. Voigt, 'The Compliance and Implementation Mechanism of the Paris Agreement' (2016) 25 Review of European, Comparative and International Environmental Law 2; and https://unfccc.int/process-and-meetings/bodies/constituted-bodies/committee-to-facilitate-implementation-and-promote-compliance-referred-to-in-article-15-paragraph-2#eq-1

48. On the work of the Compliance Committee to date, see further: https://bch.cbd.int/protocol/cpb_art34_info.shtml#cc17

49. The tools and mechanisms to enforce the Convention – an instrument of public international law – discussed above do not necessarily apply to the enforcement of contracts, which are instruments of private international law.

50. See further: http://bch.cbd.int/protocol/cpb_art34_info.shtml (accessed 15 February 2013).

51. 'Report of the Compliance Committee under the Cartagena Protocol on Biosafety on the Work of its Sixth Meeting, 4–6 November 2009, Montreal'. Available at: http://www.cbd.int/doc/meetings/bs/bscc-06/official/bscc-06-04-en.pdf (accessed 15 February 2013).

52. The Compliance Committee under the UNECE Aarhus Convention on Access to Justice, Information and Decision-Making in Decision Matters is the first to allow unrestricted access by the public to use of its compliance mechanism.

53. Articles 27 and 34.

54. Nagoya–Kuala Lumpur Supplementary Protocol on Liability and Redress to the Cartagena Protocol on Biosafety, adopted on 15 October 2010; in force on 5 March 2018, 50 ILM 105.

55. Annex II to the COP-11 decisions calls on the parties to (l) Make financial resources available with a view to supporting awareness-raising, experience-sharing and capacity-building activities in order to expedite the early entry into force and implementation of the Nagoya–Kuala Lumpur Supplementary Protocol on Liability and Redress to the Protocol; see Appendix II (Hyderabad, India, 8–19 October 2012) UNEP/CBD/COP/11/35. Available at: http://www.cbd.int/doc/decisions/cop-11/full/cop-11-dec-en.pdf (accessed 15 March 2013).

56. Article 4.

57. Article 3 (paragraph 1).

58. Although the Cartagena Protocol does provide for notification and emergency measures requirements as regards the unintentional movement of LMOs.

59. It defines damage as 'an adverse effect on the conservation and sustainable use of biological diversity, taking also into account risks to human health, that … is measurable or otherwise observable taking into account, wherever available, scientifically-established baselines recognized by a competent authority that takes into account any other human induced variation and natural variation, and is significant'.

60. There are temporal limitations to the scope of the Protocol: it applies to damage resulting from a transboundary movement of LMOs that started after the entry into force of the Supplementary Protocol for the Party into whose jurisdiction the transboundary movement was made.

61. Article 10(1).

62. According to Article 10(1), Parties 'retain the right' to provide, in their domestic legislation, for financial security. The Supplementary Protocol specifically calls for special effort to study and address the issue of financial security in the first COP-MOP (Article 10(3)).

63. Article 3(7).

64. It extends broadly to 'all GMOs'.

65. So the only main limitation in scope under the Supplementary Protocol is temporal, in that 'it shall apply to damage resulting from transboundary movement … started after the entry into force of the Protocol'.

66. An operator is defined as 'any person in direct or indirect control of the living modified organism which could, as appropriate and as determined by domestic law, include, inter alia, the permit holder, person who placed the living modified organism on the market, developer, producer, notifier, exporter, importer, carrier or supplier'.

67. See, for example, the 1963 UN Vienna Convention and the 1960 OECD Paris Convention on Civil Liability for Nuclear Accidents; and 1992 amendment to the 1954 London Convention on liability for oil pollution.

68. See generally, Pereira (2012).

69. Directive 2001/18/EC, Official Journal L106/1, 17.4.2001, repealing Council Directive 90/220/EEC OJ L117/15.

70. The legal basis of the regulation of GMOs falls upon ex-Article 37 EC (agriculture), ex-Article 154(2)(b) EC (public health) and ex-Article 95 EC (internal market) (pre-Lisbon), instead of under the environmental title.

71. The placing on the market of GM insects may also require EU approval at Part C level, although field testing to develop information for the Part C application may be conducted with only national approvals.

72. Recital 28 of the Preamble of the Deliberate Release Directive.

73. The precautionary principle is enshrined in Article 191 of the Treaty on the Functioning of the European Union (TFEU). As discussed above in the context of the Cartagena Protocol, the precautionary principle places scientific uncertainties at the centre of decision making.

74. Article 4(1) of the Deliberate Release Directive.

75. Article 2(3), ibid. 'Deliberate release' is defined under the Directive as 'any intentional introduction into the environment of a GMO or a combination of GMOs for which no specific containment measures are used to limit their contact with and to provide a high level of safety for the general population and the environment'.

76. Environmental Liability Directive 2004/35/EC, Official Journal L 143/56, 30.04.2004.

77. Environmental Crime Directive 2008/99/EC, Official Journal L 328/28, 6.12.2008.

78. The Deliberate Release Directive is one of the (over 70) pieces of Community legislation listed in Annex A of the directive, aggravated violations of which require criminalization by the Member States. Although there was a proposal by the European Parliament Committee on Civil Liberties, Justice and Home Affairs for the introduction into the Environmental Crime Directive of a specific offence of 'introduc[ing] into the environment of genetically modified organisms (GMOs) that are not approved by the European Union', this proposal was not accepted and this offence does not feature in the final text of the Directive. See the Opinion of the EP Committee on Civil Liberties, Justice and Home Affairs of 27.3.2008 ((COM(2007)0051 – C6-0063/2007 – 2007/002(COD) Draftsman: Luis Herrero-Tejedor). See further: Pereira (2015).

79. See Annex II to the Deliberate Release Directive.

80. Article 13(1) of the Deliberate Release Directive.

81. Article 19(1) of the Deliberate Release Directive.

82. See Recitals 34 and 47 of the Preamble of the Deliberate Release Directive.

83. Article 15(2) of the Deliberate Release Directive.

84. Article 4(3).

85. Available at: http://europa.eu/rapid/pressReleasesAction.do?reference=MEMO/06/61 (accessed 23 March 2012).

86. The system of approval and authorization is elaborate and complex, requiring the intervention of the European Food Safety Authority (EFSA). There has been indeed over the years a *de facto* moratorium on approvals, and while only a few GMO foods have been approved, some Member States, invoking the precautionary principle, have prohibited the marketing even of approved GMOs (Nucara, 2003, p. 47).

87. Available at: http://www.wto.org/english/tratop_e/dispu_e/cases_e/ds293_e.htm (accessed 23 March 2012).

88. In 2003, the WTO case launched by the USA, supported by Canada and Argentina, sought to oppose the EU's authorization regime for GMOs, since these countries are major producers of GMOs. In 2006, the WTO Panel ruled that the EU's authorization regime had acted as a *de facto* moratorium on importation of GMOs into the EU from 1999 to 2003. From 2008 to 2010, the parties altered the 'reasonable period of time' for implementation of the recommendations and rulings of the WTO Dispute Settlement Board more than ten times, as the EU appealed for a time extension. Eventually, the parties agreed to a 'mutually agreed solution' therefore potentially weakening the WTO panel's recommendations. See further: https://www.wto.org/english/tratop_e/dispu_e/cases_e/ds291_e.htm

89. Regulation 1829/2003 on Genetically Modified Food and Feed [2003] OJ L 268/1.

90. There have been to date no applications for GM insect authorization for 'placing on the market' in the EU. Further information, including details of applications for field trials (deliberate release) in the EU, can be found at: http://gmoinfo.jrc.ec.europa.eu (accessed 10 November 2021).

91. Oxitec made an application to the Spanish National Authorities under Ley 9/2003 (the implementation of part B of directive 2001/18/EC in Spain) in late 2012. But the Spanish authorities felt that they could not authorize the trial without additional data and significant containment measures in place. In 2015 a second regulatory submission was made, but this application was withdrawn by the applicants due to concerns remaining regarding the confinement of the trial site. See further House of Lords (2015–2016) G. Turner, C. Beech and L. Roda, 'Means and ends of effective global risk assessments for genetic pest management', *BMC Proceedings* 2018, 12(Suppl. 8):13.

92. Yet the conditions set by the importing state are subject to the limitations imposed by WTO law.

93. Not only the free trade provisions of the WTO Agreement, but also there are potential conflicts with the Agreement on the Application of Sanitary and Phytosanitary (SPS) Measures; the Agreement on Technical Barriers to Trade; and the 1995 Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS). The Vienna Convention on the law of Treaties (1969) deals with the question of conflicts between international treaties. On this question, see Qureshi (2000).

94. Yet the parties under Cartagena agreed that trade and the environment should be mutually supportive, that the Protocol should not be interpreted as implying a change in the rights and obligations under existing agreements, and that it should not be subordinated to other agreements.

95. Article 10(6) of the Cartagena Protocol. Under WTO law, states must adopt the least trade restrictive measures available.

96. Although not discussed in this chapter, the interests and concerns of transit countries must also be addressed (see Article 6 of the Protocol).

References

Beyerlin, U. and Marauhn, T. (2011) *International Environmental Law*. Hart Publishing, Oxford, UK.

Birnie, P., Boyle, B. and Redgwell, C. (2009) *International Law & the Environment*, 3rd edn. Oxford University Press, Oxford, UK.

CBD (1992) *Convention on Biological Diversity* (adopted 5 June 1992, entered into force 29 December 1993). United Nations Treaty Series (UNTS), Vol. 1760. Available at: treaties.un.org/doc/Publications/UNTS/Volume%201760.pdf, accessed April 2022. [English text, pp. 143–169.]

CBD (2010) *Strategic Plan for Biodiversity 2011–2020, including Aichi Biodiversity Targets*. Convention on Biological Diversity, Montreal, Canada. Available at: https://www.cbd.int/doc/strategic-plan/2011-2020/Aichi-Targets-EN.pdf (accessed 28 April 2022)

CBD (2016) *Guidance on risk assessment of living modified organisms and monitoring in the context of risk assessment*. UNEP/CBD/BS/COP-MOP/8/8/Add.1. Convention on Biological Diversity, Montreal, Canada.

CBD (2017) *Report of the ad hoc technical expert group on synthetic biology, CBD/SYNBIO/AHTEG/2017/1/3*. Convention on Biological Diversity, Montreal, Canada.

CBD (2021) First Draft of the Post-2020 Global Biodiversity Framework. Convention on Biological Diversity, Montreal, Canada. Available at: https://www.cbd.int/doc/c/914a/eca3/24ad42235033f031badf61b1/wg2020-03-03-en.pdf (accessed 28 April 2022)

CITES (1973) Convention on International Trade in Endangered Species of Wild Fauna and Flora. *UNTS* 993(I), 14537. [Adopted 3 March 1973, entered into force 1975.] Available at: https://treaties.un.org/doc/publication/unts/volume-993-i-14537-english.pdf (accessed 28 April 2020)

ECD (2021) Proposal for a Directive of the European Parliament and of the Council on the protection of the environment through criminal law and replacing Directive 2008/99/EC, Brussels, 15.12.2021 COM(2021) 851 final

Eckersley, R. (2004) The Big Chill: The WTO and Multilateral Environmental Agreements. *Global Environmental Politics* 4, 2.

Hill, J., Johnston, S. and Sendashonga, C. (2004) Risk Assessment and Precaution in the Biosafety Protocol. *Review of European Community and International Environmental Law (RECIEL)* 13, 263–269.

House of Lords (2016) *Genetically modified insects*. 1st Report of Session 2015–16. HL Paper 68. House of Lords Science and Technology Select Committee, London.

Koester, V. (2013) The Compliance Mechanism of the Cartagena Protocol on Biosafety: Development, Adoption, Content and First Years of Life. In: Segger, M.C. and Perron-Welch, C. (eds) *Legal Aspects of Implementing the Cartagena Protocol on Biosafety*. CUP, Cambridge.

Lee, M. (2008a) *EU Regulation of GMOs: Law and Decision Making for a New Technology*. Edward Elgar Publishing, Cheltenham, UK.

Lee, M. (2008b) The governance of coexistence between GMOs and other forms of agriculture. *Journal of Environmental Law* 20, 193–212.

Lefeber, R. (2012) The Legal Significance of the Nagoya-Kuala Lumpur Supplementary Protocol: The Results of a Paradigm Evolution. Amsterdam Law School Legal Studies Research Paper No. 2012-87. University of Amsterdam, Netherlands.

Lin, A.C. (2017) Mismatched regulation: genetically modified mosquitoes and the coordinated framework for biotechnology. *University of California Davis Law Review* 51(1), 205–232.

Mackenzie, R. (2004) The Cartagena Protocol after the First Meeting of the Parties. *Review of European Community and International Environmental Law* 13, 270–278.

Mackenzie, R., Burhenne-Guilmin, F., La Viña, A.G.M. and Werksman, J.D. (2003) *An Explanatory Guide to the Cartagena Protocol on Biosafety*. IUCN Environmental Policy and Law Paper No. 46. *IUCN Environmental Law Centre*, The World Conservation Union. Thanet Press, Cambridge, UK.

Marshall, J. (2011) Commentary: The Cartagena Protocol in the context of recent releases of transgenic and *Wolbachia*-infected mosquitoes. *Asian Pacific Journal of Molecular Biology & Biotechnology* 19, 93–100.

McGraw, D.M. (2002) The CBD – Key characteristics and implications for implementation. *Review of European Community and International Environmental Law* 11, 17–28.

Nijar, G. (2012) The Nagoya–Kuala Lumpur Supplementary Protocol on Liability and Redress to the Cartagena Protocol on Biosafety: An analysis and implementation challenges. *International Environmental Agreements: Politics, Law and Economics* 13(3), 271–290. doi: 10.1007/s10784-012-9187-9

Nucara, A. (2003) Precautionary Principle and GMOs: Protection or Protectionism? *International Trade Law and Regulation* 9, 47–53.

Pereira, R. (2012) Compliance and enforcement in international, European and national environmental law. In: Makuch, K. and Pereira, R. (eds) *Environmental and Energy Law*. Wiley-Blackwell, Oxford, UK.

Pereira, R. (2015) *Environmental Criminal Liability and Enforcement in European and International Law*. Brill–Nijhoff, Leiden, Netherlands.

Qureshi, A.H. (2000) The Cartagena Protocol on Biosafety and WTO – Co-existence or Incoherence? *International and Comparative Law Quarterly* 49, 835–855.

Sands, P. and Peel, J. (2012) *Principles of International Environmental Law*, 2nd edn. Cambridge University Press, Cambridge, UK.

Swindendenhammer, S. (2020) The Rise, Regulation and Risks of Genetically Modified Insect Technology in Global Agriculture. *Science, Technology & Society* 25(1), 124–141.

Telesetsky, A. (2011) The 2010 Nagoya-Kuala Lumpur Supplementary Protocol: A New Treaty Assigning Transboundary Liability and Redress for Biodiversity Damage Caused by Genetically Modified Organisms. *The American Society of International Law: Insight* 14, 1–11.

Tsioumani, E. (2010) Liability and Redress: Supplement to the Cartagena Protocol. *Environmental Policy and Law* 40, 293.

Turner, G., Beech, C. and Roda, L. (2018) Means and ends of effective global risk assessments for genetic pest management. *BMC Proceedings* 12 (Suppl. 8), 13.

UN (1980) Vienna Convention on the Law of Treaties, 23 May 1969, entry into force 27 January 1980. *United Nations Treaty Series (UNTS)* 1155, 33.

UN (1989) 1989 Basel Convention on the Transboundary Movement and Disposal of Hazardous Wastes, adopted on 22 March 1989; in force on 5 May 1992, 1673 UNTS 5.

UN (1998) Rotterdam Convention on the Prior Informed Consent Procedure for Certain Hazardous Chemicals and Pesticides in International Trade, adopted on 10 September 1988, in force 24 September 2004, 2244 UNTS 337.

WHO (2014) *Guidance Framework for Testing of Genetically Modified Mosquitoes*, 1st edn. World Health Organization, Geneva, Switzerland.

WHO (2021) *Guidance Framework for Testing of Genetically Modified Mosquitoes*, 2nd edn. World Health Organization, Geneva, Switzerland.

28 Risk Analysis of Transgenic Insects

Keith R. Hayes[1] and M.M. Quinlan[2]*

[1]*CSIRO, Data61, Hobart, Australia;* [2]*Centre for Environmental Policy, Imperial College London, UK*

28.1 Introduction

28.1.1 Scope of this chapter

In the years that have elapsed since the first edition of this chapter, research and development into transgenic insects has continued and been accelerated by the discovery that CRISPR/Cas systems could be directed to perform site-specific DNA cleavage and drive homology-directed repair (Cong *et al.*, 2013; Mali *et al.*, 2013; Concha *et al.,* 2016) (Concha and Papa, Chapter 7, this volume), paving the way for a new class of easily programmable homing endonucleases. The Clustered Regularly Interspaced Short Palindromic Repeats, or CRISPR, and the associated protein/Cas systems have now taken their place alongside other types of homing endonucleases (HEGs, ZFNs, TALENs), various forms of meiotic drives and inheritable genetic sterilization methods (Burt, 2014; Lindholm *et al.*, 2016) that together constitute a diverse array of methods for producing genetic modifications in insects that sit on a continuum from non-driving, self-limiting constructs to (theoretically) very low-threshold, self-sustaining gene drives (see Chapters 8–12, this volume).

The purpose of these modifications is still varied and includes laboratory research in gene regulation. Our focus, however, remains transgenic insects that are intended to be released into the environment in order to control agricultural pests or vectors of human and animal pathogens, and the risk analysis processes that support such releases. Box 28.1 describes a niche use of genetic modification for pest control.

Since the first edition of this volume was published in 2014, several more field releases of transgenic insects have taken place, including: (i) sustained releases of a sterile male strain (OX513A) of *Aedes aegypti* mosquitoes in Brazil (Carvalho *et al.*, 2015); (ii) field trials of a 'self-limiting' strain of the diamondback moth (*Plutella xylostella*) genetically engineered to allow the production of male-only offspring (Shelton *et al.*, 2020); (iii) a single field release of a transgenic sterile male strain of *Anopheles coluzzii* mosquitoes in Burkina Faso (Pare Toe *et al.*, 2021); and, most recently, (iv) field releases of a female-lethal strain (OX5034) of transgenic male mosquitoes in the Florida Keys (Waltz, 2021). Studies in the laboratory run apace, to prepare for potential field programmes of the future (e.g., Kandul *et al.*, 2021).

---

*Corresponding author, email: m.quinlan@imperial.ac.uk

Box 28.1. Modifications to support production of insects for field use.

For insects destined for field use, the purpose of genetic engineering is generally to produce a phenotypic characteristic that will enhance the use of the mass-reared population to be released. There are other possible purposes, however, which would impact the risk profile that will merit different treatment in risk management decisions. One such purpose would be to introduce a marker gene to facilitate identification of a released population, or its strain, as a cost-effective support for monitoring and surveillance.

Another purpose is to enhance the production process, which has become increasingly recognized as a field of research and impact in itself (Leppla and De Clercq, 2019). Key among these enhancements is to create a sexing strain, so that the males and females can be separated at lower cost and with more accuracy than is achieved through manual sorting. For field programmes that rely on release of a single sex (usually male only) the cost savings from this is significant. Presently, the only field programme studying this option is the multinational sterile insect control of the New World screwworm (*Cochliomyia hominivorax*) discussed further in Scott *et al*., Chapter 17, this volume. The advantages of a transgenic line for production are discussed by Concha *et al*. (2016), but to date it appears the lack of effective monitoring for male-only releases of this livestock pest has limited the use of this strain. However, future examples of genetic engineering that are aimed at operational or production efficiencies might be considered differently if the modification has no impact on characteristics generally considered in risk analysis for field release.

This chapter draws upon the risk assessments (defined here as the process of risk identification, characterization and evaluation) and risk analyses (defined here as an overall process comprising risk assessment, management and communication) that supported these releases, and the commentary associated with these, together with experiences with similar living products that are not transgenic. The first edition of this chapter considered the different approaches to risk for two conditions of use: limited by containment or confinement; and open field release. In this update, we approach the risk analysis issue from the perspective that containment lies on a more gradual continuum influenced by geographical location, potential exposure to wild mating populations, timing and time phase of releases, and other factors. As previously observed, the traditional paradigm of managing risk by preventing or avoiding interaction with biodiversity in the environment is no longer valid when the objective of the release relies on interaction and possibly persistence in the environment.

28.1.2 Historic context for biosafety risk analysis and regulation

Although transgenic *Drosophila* were among the early model species of genetically modified organisms for research in the laboratory (Spradling and Rubin, 1982), it was some time before other insect species were transformed (Handler, 2002) (O'Brochta, Chapter 1, this volume). The first applications for large-area commercial use of genetically modified organisms were for crop plants. This occurred at a time when the public was much more informed and interested in environmental quality in general, in line with an awakening to the potential impacts from chemical pesticides. Methods for environmental impact assessment were well developed and broadly applied. Some experts consider that this historic context led governments to apply a higher environmental standard to the assessment of genetically modified crops than had been incorporated into regulation of earlier methods of pest control (NRC, 2002). The initial policy in the USA was that the product resulting from biotechnology, not the method of development, was to be assessed for risk under existing laws (OSTP, 1986). Other regulatory frameworks for genetically modified organisms (GMOs), however, arose from the perception that there was something inherently dangerous about the method of achieving a novel trait, rather than by focusing on the characteristics of novel traits themselves.

While the application of risk analysis to support decision making around biological interventions is widespread, the exact approach varies and is influenced by divergence of

cultural context regarding who has the burden of proof – and what the endpoints are. The originating assumptions have a significant effect on the conclusions drawn from a risk analysis, if not also on the analysis itself. Hayes (2004) discussed the various interpretations of risk and what influences it. The use of familiarity and substantial equivalence, first published by the Organisation for Economic Co-operation and Development (OECD), preceded the Cartagena Protocol on Biosafety to the Convention on Biological Diversity (CBD) coming into force (UNEP/CBD, 2000). The use of substantial equivalence can give direction and increase efficiency in the burdensome process of case-by-case decision making. This approach would be subject to opposition later, causing a greater burden to the regulatory process, but is an intellectual predecessor for recent rigour in establishing pathways to harm before including concerns in a formal risk analysis (e.g., Roberts *et al*., 2017; Connolly *et al*., 2021).

Certainly, the concurrence of the Conference on Environment and Development, or 'Earth Summit' (UNCED, 1992), and its subsequent influence on the CBD and its Cartagena Protocol (discussed by Pereira, Chapter 27, this volume) has had a significant influence on the regulation of biotechnology and use of risk analysis. Following the entry into force of the Cartagena Protocol, the majority of countries have opted for new regulation and even legislation for ensuring the safety of products of biotechnology – to the exclusion of other methods of obtaining novel traits. These legal instruments follow the guidance in the Protocol, while at times requiring more, or perhaps adding greater, demands by including optional aspects as if they were obligatory.

The creation of national biosafety frameworks has been a priority under international funding schemes (Johnston *et al*., 2008; McLean *et al*., 2012), with approximately 130 developing countries receiving support towards this aim. These frameworks arose from special funding schemes rather than from years of experience and resulting regulatory adjustment and revision. Perhaps because of this focus on the method of development of the products, it is not unusual for biotechnology

regulation to be applied in parallel with other regulatory frameworks more suited to the specific use of the organism or novel trait. There are also numerous cases in which a country has prepared a legislative and regulatory framework yet remained hindered by 'implementation gaps' (Bulkeley *et al*., 2013) that prevented their opportunity to gain any benefits from biotechnology products (Nuffield Council on Bioethics, 2004). National and regional frameworks reflect the cultural context of the scientific and societal climate, as seen in reviews by such as the European Food Safety Authority (EFSA, 2020).

Simultaneously, multilateral initiatives such as those developed under the Codex Alimentarius Commission, the World Organization for Animal Health (OIE), or the International Plant Protection Convention (IPPC), and for health issues the World Health Organization (WHO), have achieved some harmonization in regard to recommended practices for risk analysis of GMOs, due to the clear scope of each (FAO, 2011), coordination across authorities (e.g., FAO, 2021) and common objectives of the contracting parties. These sources include categories of potential hazards or harms within each authority to consider in regard to biotechnology but are generally conceptual (as outlined in Quinlan, 2014, first edition of this chapter). The Convention on Biological Diversity (https://www.cbd.int/convention/text/, accessed April 2022) itself has sought further clarity on the use of risk assessment in relation to GMOs (or living modified organisms (LMOs) as defined in the Cartagena Protocol). Other more detailed guidance on risk analysis is given by other multilateral organizations, based on years of discussion and consultation, but not necessarily limited to biosafety (e.g., OECD, 1986, 1993, 2005; UNIDO, 1995; UNEP, 1996, 2016).

Finally, what might be referred to as soft regulation has continued to have a significant influence with the global research community. Researchers working with transgenic insects have been highly aware of the need to be cautious in moving out of confinement (physical, biological, or environmental) to open field until broader agreement could be achieved on methodologies for decision

making, among other things. They have, to a large degree, voluntarily self-regulated through discussion, collaboration, publishing articles evoking caution and peer review. This long-running stance is reflected in reports from the WHO Special Programme for Research and Training in Tropical Diseases (TDR) convened meetings on genetic strategies against vector mosquitoes (WHO, 1991, 2010; Takken *et al*., 2002; Knols and Louis, 2006) and in various other specialty conferences such as the Vector Biology Network (Beaty *et al*., 2009). Indeed, some researchers find that official regulation of biotechnology and their definitions of what transgenesis entails cannot keep pace with scientific discovery so that the oversight of institutional governance and peers is becoming even more important (O'Brochta *et al*., 2020).

This guidance is developed through independent committees, in workshop settings or as part of projects. The Brazil branch of the International Life Sciences Institute (ILSI) published a Spanish version of guidance on risk assessment of a range of GMOs with input from authors throughout the Americas (De Andrade *et al*., 2012). Published guidance on testing genetically modified (GM) mosquitoes, for example, was established as a result of the TDR-convened consultations, in alliance with the Foundation for National Institutes of Health (FNIH), in 2014 with a substantial update in 2021 (WHO, 2021). Agreement on conditions for use of gene drives, for example using GM insects for vector control, has been sought, resulting in various publications on best practice (James *et al*., 2018, 2020; Annas *et al*., 2021). Further highlights of global and regional efforts to coordinate regulatory approaches to GM insects are discussed by Beech *et al*., Chapter 25, this volume.

Throughout the history of genetic modification of organisms, one feature universally present in biosafety guidance and frameworks is the use of risk assessment as a legitimate decision-support methodology, which also provides the opportunity for transparency in the scientific reasoning applied. Risk assessment, perception of risk and regulation of GMOs are intricately linked. Simply avoiding risks identified in this process is not

the intention of the approach, as discussed further in Beech *et al*., Chapter 25, this volume.

Risk analysis goes further to capture residual uncertainty or risk after applying management proportional to the risk as assessed; acceptance of risk in relation to the benefits and distribution of benefits; and to some degree the political appetite to take decisions in the face of uncertainty. This makes risk analysis a valuable tool for numerous activities using transgenic insects, including planning research, project implementation, product roll-out, and investment decisions, which go far beyond formal government regulation, but which are continually affected by it.

28.2 Risk and the Risk Assessment Process

The word 'risk' invokes situations that entail uncertain adverse outcomes. Risk is a phenomenon that reveals itself through time if we carefully observe and record adverse outcomes and the circumstances under which they occur, such as the number of fatal car accidents last year in Australia. With this information we can identify how the probability of adverse outcomes is influenced by different factors, such as the age of the drivers involved in fatal accidents, and infer the benefits of risk management activities, such as the imposition of new speed limits, or the effect of a new drunk-driving campaign.

Risk assessment is a process that looks to the future and predicts how often adverse outcomes will occur and how severe these outcomes will be. We use these predictions to guide decisions about the costs, benefits and acceptability of new technologies and new risk management activities, as part of the risk analysis process. Risk predictions are most useful for this purpose when they are carefully contextualized, that is when the spatial and temporal scope, and the factors that influence the risk estimates, are clearly described, and the risk estimates are expressed in terms of measurable outcomes with well-defined units. This allows the predictions to be compared with outcomes and the effect

of different factors and risk management activities to be correctly attributed.

Kaplan and Garrick (1981) stated that risk analysis seeks to answer three questions: (i) What can go wrong? (ii) How likely is it that that will happen? and (iii) If it does happen, what are the consequences? Under the definitions used here these three steps are the fundamental components of a risk assessment and in a risk analysis they would be followed by additional management and communication activities. These three steps, and the distinction between risk analysis and risk assessment used here (and elsewhere but not universally – see for example Rausand and Haugen, 2020), are reflected in many of the frameworks and risk guidance documents that are relevant to, or specifically designed for, transgenic organisms, including insects.

In addition to the fundamental steps, Kuzma (2019) suggested that risk assessments for genetically engineered organisms should be conducted in accordance with five principles to help ensure that the process is 'procedurally robust'. Some of these principles emphasize the importance of providing stakeholders and potentially affected communities with the opportunity to contribute to the risk analysis process, or even earlier in the process by contributing around viable alternatives to a new technology proposal (see also Stirling *et al.*, 2018; Hartley *et al.*, 2019). These are not new suggestions; similar sentiments have been expressed for many years (NRC, 1996), but the large number of publications and guidance documents in which they now feature so prominently is a novel aspect of the current risk analysis discourse around transgenic insects.

This definition of risk analysis presumes the availability of risk acceptance criteria. If the estimated risk is acceptable, compared with other existing methods for pest control for example, or if management measures can reduce the risk sufficiently, then the process continues and risk assessment informs the risk management plan. To be scientific, risk predictions, including the effect of the management, are monitored and findings fed back into the original (or subsequent) assessment, making the method an iterative learning process rather than a static document or dossier.

In engineering contexts, risk acceptance criteria are typically specified in terms of the maximum permissible frequency of accidents or fatalities per year, or in terms of the minimum reliability of critical safety components, and these may be codified in industry standards or guidelines published ahead of, or independently from, individual risk assessments. Equivalent criteria can also be found in ecotoxicological contexts. In Australia, for example, the Victorian State Environmental Protection Policy (Waters) implements risk-based national water quality guidelines by specifying the acceptable quantiles of contaminant concentrations in different water bodies (Victorian Government Gazette, 2018).

In the context of transgenic insects, minimum standards for containment are specified when research and development are laboratory-based and in many cases this obviates the requirement for a formal risk assessment (Box 28.2). If the research clearly fits a risk categorization, it avoids the case-by-case approach and is instead considered based on the recommended level of containment. The focus is on ensuring containment, rather than on uniqueness of the organism.

For field releases of transgenic insects and other biological risk sources, however, there are often no equivalent standards that can serve as risk acceptance criteria. The risk analysis process nonetheless requires that these be specified. Ideally this should occur before or immediately after the identification of the values (the risk assessment endpoints) that are perceived to be threatened by the risk source (Hayes *et al.*, 2007), but certainly before the related risk analysis is concluded.

In practice, acceptance criteria might not be explicitly stated and decisions regarding acceptability are left to the discretion of, and internalized within, the relevant biosafety authorities. Alternatively, acceptance criteria may be specified explicitly but in a qualitative manner and therefore possibly interpreted by different parties in different ways.

Hayes *et al.* (2013) suggested that a key challenge with biological risk sources, and a contributing impediment to the specification of acceptance criteria, is uncertainty surrounding the potentially complex ecosystem impacts.

Box 28.2. Risk assessment for contained or confined use of a GMO.

The spread and persistence of genetically modified insects can be prevented by molecular (genetic), ecological, geographical, climatic, reproductive or barrier (physical confinement) methods used initially in combination to provide independent lines of defence against premature release (Akbari *et al*., 2015), but ultimately relaxed gradually to permit a phased-release strategy (WHO, 2021).

Whereas the focus of the risk assessment and analysis for small-scale confined, or large-scale unconfined, field releases of transgenic insects is the safety and possible impact of the organism, the primary question in physical confinement is the probability and consequences of escape or accidental release from the containment facility. In fact, in many instances the risk associated with the organisms is not considered in depth, but rather the fail-safe nature of the containment is evaluated. (For exceptions requiring a comprehensive assessment, see NIH, 2019.)

This, then, becomes an evaluation or assessment of the management measures for construction, maintenance and operation of a containment facility. Furthermore, as many countries have established regulations or guidance regarding containment facilities, the risk assessment process may be replaced largely by assurance of compliance with these existing requirements.

Containment requirements are generally presented in terms of levels assigned by a combination of criteria, such as discussed in the Arthropod Containment Guidelines developed by the American Committee of Medical Entomology (ACME, 2019), and recently updated for gene drive-modified arthropods (ACME/ASTMH, 2021). An important distinction for this approach is that assignment of a particular level of containment does not necessarily equate with an equivalent probability of harm associated with the organism in question.

Containment is used for study of novel organisms to a large degree because of the uncertainty or lack of knowledge about the risk factors, rather than because of some acknowledged 'danger' from the organisms (Hilbeck and Meier, 2006). This is a 'precautionary approach' widely used for international trade of biological control agents which were, for example, subjected to isolation for one to three generations in a quarantine facility. In this case, the requirements also allowed for detection of any contamination of the biocontrol agents with parasites or infectious agents.

Also, the evaluation of risk in regard to worker and researcher safety may require yet further assessment of the risk associated with the study organism. Indeed, as exposure increases in the laboratory setting certain risks, such as those from an allergic reaction, could increase. These considerations are only affected by genetic engineering if this results in a change of some characteristic of the insect, and should not be mistaken as protecting against exposure to transgenic insects *per se*.

Kuzma (2019) also cited the high uncertainty associated with 'emerging risks', such as the open release of GM insects, as a key motivation for procedurally robust risk assessment principles, which stipulate *inter alia* the need to consider the acceptability of risk estimates to those who provide input to the analysis.

Various forms of uncertainty occur in risk analysis and risk assessment (Regan *et al*., 2002; Spiegelhalter and Riesch, 2011). Outside of linguistic uncertainty (which is a prominent factor in qualitative risk assessments) the two key sources are natural variation, which can be better characterized with additional research and observation but not reduced, and epistemic uncertainty (lack of knowledge or information), which can possibly be reduced by further research. The opportunity to clearly indicate and record where scientific evidence is conclusive and where uncertainty remains high is one reason that risk assessment and risk analysis have continued to be universally applied in governmental decision making.

Additional risk management and monitoring may be used when uncertainty is high, during the process of answering some of these questions. A decision maker's response to uncertainty is at the heart of the often cited 'precautionary principle', discussed further by Pereira, Chapter 27 this volume. Various stakeholders interpret this principle in various ways, as Beech *et al*. discuss in Chapter 25, this volume. Certainly, there was not uniform agreement in the approach now enshrined in Annex III of the Cartagena Protocol (Kapuscinski, 2002). The risk analysis process supports making a decision that could include additional management or precaution due to uncertainty. In general,

sovereignty remains in terms of risk acceptance or aversion, although transparency and consistency are global principles.

28.3 Risk Analysis for Transgenic Insects

Risk analysis is a widely used method for formulating and facilitating decisions regarding alternative actions for control of insect pests (Hutchison *et al.*, 2006; EPPO, 2011; FAO, 2017a, 2019). There are different attributes to risk analysis when the intervention involves intentional introduction of living organisms into the environment. In agriculture, such interventions include the introduction of biological control agents (classical and augmentative), pollinators, mycofungicides and other biopesticides. Another example is sterile insect technique (SIT), which involves the release of living insects as a means of targeting a compatible breeding population in the local environment.

Most studies and guidance on risk analysis, ecological risk assessment or environmental impact assessment of an intentional release of living insects are referring to an open field release. Accepted best practice requires a phased approach to reduce risk during the testing, development and evaluation of an insect 'product', and thereby ensures safety and efficacy prior to use in an ongoing programmatic application. The typical chain of events (Fig. 28.1) is to conduct studies related to basic proof-of-principle and to safety in the laboratory, and then to continue to a confined release.

Multilateral guidance on GM organisms and gene drive-modified organisms, largely published since the first edition of this book,

typically describe risk analysis as a series of steps that address the following issues:

- protection goals, values and problem formulation;
- characterization of the GM organism and receiving environment;
- risk calculation and characterization; and
- risk management, communication, acceptability and monitoring.

The risk analysis should be embedded within, and its predictions inform, a phased release strategy. The use of such a strategy has been a consistent feature of biosafety guidance for several decades, and most recently reinforced by the World Health Organization in the context of genetically modified mosquitoes (WHO, 2021).

The phased approach shown in Fig. 28.1 applies to development of GM insect products destined for large-scale programmatic release. Benedict *et al.* (2008) expounded on the importance of the phased approach for GM insects and suggested that a cage trial phase is imperative for GM insects containing a self-sustaining 'drive system'. During a phased release strategy, with confinement provided by sterility, for example, or some fitness-reducing characteristic that results in similar mortality in the field, the necessity for additional confinement strategies such as physical containment in small cages can be relaxed in order to allow experiments to be conducted in larger, more natural field settings.

The advantage of larger, more natural open field studies is to observe critical parameters (such as fitness parameters) in more realistic settings, to more realistically determine efficacy of the intervention, which can be challenging to predict from cage studies alone (Robert *et al.*, 2013; Mumford *et al.*, 2018), and to reveal ecological interactions which may not arise in the laboratory or

Fig. 28.1. Phases in assessment of a transgenic insect.

cage, or in a geographical and climatic area that is not within the natural distribution of the insect species. Benedict and Robinson (2003) proposed that sterile transgenic insects should be the first for field studies in order to gain this type of knowledge while preventing persistence in the environment, but it is recognized that ultimately the phases between steps are less clear when the transgenic insects are intentionally designed to persist. Risk analysis and regulatory review may be needed earlier if a field trial will essentially become a programmatic intervention when designed to persist.

Many countries are not yet resourced to be developing and mass-producing transgenic insects. Insects with a genetic marker, for example, could eventually be purchased through the international channels existing for sterile insect technique (described in Quinlan and Enkerlin, 2003; Quinlan and Larcher-Carvalho, 2007; Enkerlin and Zavala-Lopez, 2017). Current trials with GM mosquitoes have required international shipment of transgenic eggs (e.g., Government of Malaysia 2010, 2013a,b). Therefore, an initial event to trigger assessment may be the request to import a transgenic insect (in whatever life stage) into a country. Decisions relating to risk for international trade in insects are notoriously variable and gaps remain in terms of authorities and guidance (OIE, 2022). Alternatively, if the transgenic living product would be created domestically, a different type of permit and assessment such as certification of a containment facility may be required. Figure 28.2 shows some of the assessment points in the phases also shown in Fig. 28.1, with the example of containment facilities as the chosen confinement step. The steps would be typical for introducing a novel product into the market or into a public programme.

A phase not shown in Fig. 28.2 is the scaling-up of production of the transgenic insect. This entails a related series of operational and risk assessments: site selection for that facility or activity, review of safe transfer from the facility to release sites, quality assurance regimes, worker safety, financial risk, etc. This is overlapping but distinct from the requirements for site selection for an outdoor
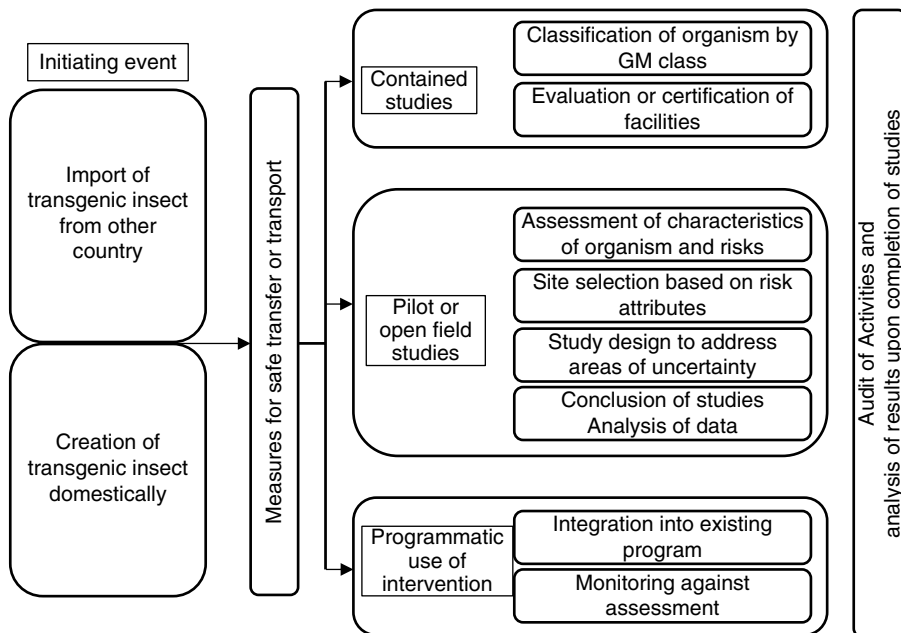
Fig 28.2. Phases in assessment, testing and approval of a transgenic insect for use in an ongoing programmatic intervention for pest or vector control.

cage or pilot field study (e.g., Brown *et al.*, 2014). Discussion of some of these issues appears in a model business plan for mass-rearing facilities, written in relation to irradiated insects (IAEA, 2008).

For each phase, a thorough analysis of results against the original assumptions in the risk assessments is required. Ideally, if similar studies are carried out over time, the data will begin to reduce uncertainty. Similarly, additional experiments may be carried out to specifically address uncertainty or concerns that arose in the Hazard Identification stages (for example, although not for GM insects, see Popovici *et al.*, 2010 for study responsive to expressed concerns).

28.3.1 Protection goals, values and problem formulation

Risk assessments are performed to protect human values. The values are reflected in the risk assessment's protection goal. The goals may be drawn from national and international legislation and may also be specified at quite a high level. For example, the African Union's Agenda 2063 (available at: https:// au.int/agenda2063/goals (accessed 28 April 2022)) specifies a goal of 'Environmentally sustainable and climate resilient economies and communities' with a priority area of 'biodiversity conservation'.

To be actionable within a risk-based decision-making framework, broad protection goals such as these need to be translated into more specific assessment endpoints, that describe an entity – typically at a population level for an ecological value, or at an individual level for a human health value – and an attribute, such as mortality, morbidity, or reduction in abundance (Suter *et al.*, 2005).

The assessment endpoints, and the chain of events, causal pathways or 'pathways to harm', that link exposure to a risk source, such as the field release of a genetically modified insect, and adverse effects on the endpoints are identified and described in the 'problem formulation' stage (Wolt *et al.*, 2010). Detailed guidance on the steps involved at this initial stage of a risk assessment are available in the literature (Devos *et al.*, 2019)

together with examples of pathways to harm for transgenic insects (Connolly *et al.*, 2021).

The identification of pathways to harm during the problem formulation stage of a risk assessment is equivalent to the initial hazard identification stage under the popular United States Environmental Protection Agency (USEPA) framework (USEPA, 1998) for ecological risk assessment. Raybould (2020) emphasized that this stage should not be an 'open ended search' for all possible hazards but rather a definition of the mechanisms by which the risk source may lead to clearly defined harms. For example, horizontal gene transfer is an often-cited hazard, but it is not a pathway to harm unless the transfer leads to some negative manifestation or impact (see the discussion in FAO/IAEA, 2006; Keese, 2008).

How 'clearly defined harms' are enunciated for novel technologies, however, is not always obvious. Lists of potential harms associated with the field release of transgenic insects are currently available in the literature (see for example EFSA GMO Panel, 2020), but in any real application these lists should be augmented by the concerns expressed by the relevant stakeholders and communities who could be exposed to the release. Furthermore, Hayes *et al.* (2018a) cautioned against over-reliance on checklists and identified a variety of techniques to help analysis identify what might go wrong with the application of a novel technology. Similarly, the Scientists' Working Group on Biosafety (1998) developed flow charts for decision support for a variety of possible GMOs. These flow charts are designed to ensure that all considerations have been addressed, so that conclusions drawn for a particular assessment do not sway decisions for other assessments without review of other potentially relevant factors.

The effect of the transgenic traits on integration into an operational control programme, as well as worker safety issues during research and for the production facility that will be needed, are additional points of consideration in any assessment, because the purpose of most research on pest species ultimately will be to improve control efforts in an ongoing programmatic intervention (Hoy, 2000; James, 2005).

Hoy (2000) concluded from her own experience that the efforts for introducing a transgenic insect into a pest control programme can be divided into three similarly demanding phases: the planning and technological development of the product; field testing; and the integration of the product into a pest management programme. This last phase includes public education, cost-effective mass rearing, and additional research on possible hazards when conducting large-scale releases over longer time periods.

It is also worth noting at this point that several authors (Nelson *et al.*, 2007; Finkel *et al.*, 2018; Stirling *et al.*, 2018) call for risk analysis to address a clearly defined problem rather than a specific (in this case genetic) solution, and thereby assess the risks, benefits and opportunities offered by a range of possible solutions to the problem, including the solution(s) currently used to address it. As Finkel *et al.* (2018) noted, this point of view interprets the problem formulation step in a risk assessment quite differently from the interpretation presented in USEPA

guidance documents, and it is also much broader than that represented in the multilateral guidance documents on risk assessment relevant to transgenic insects. This type of analysis is also much broader than that typically conducted (or requested) by national biosafety agencies when considering individual applications to release genetically modified insects, due to the process's reliance on an applicant or possible developer of the technology and for private investment scenarios, often with commercial aims.

28.3.2   Characterization of the GM organism and receiving environment

Insects can have a range of characteristics that affect control programmes and should be considered in a risk assessment for transgenic insects, including:

- short generation time;
- mobility and ability to disperse (Box 28.3);

Box 28.3. Mobility of transgenic insects

The North American Plant Protection Organization (NAPPO) regional standard (RSPM No. 27) Guidelines for Importation and Confined Field Release of Transgenic Arthropods in NAPPO Member Countries (NAPPO, 2007), which is now archived, was possibly the first official guidance specific to transgenic insects. It addressed any transgenic insect with possible impacts on plant health under consideration for import and confined field release in the three member countries (Canada, USA and Mexico). This could include biocontrol agents or other beneficial insects.

The possible mobility of insects, in contrast to GM plants, for example, poses some additional biosafety considerations. It is difficult to anticipate the impact of dispersal capacity in general regulations. NAPPO (2007) also noted the dispersal ability of a transgenic insect as a factor for consideration in risk assessment.

The regulation of (non-transgenic) butterfly releases in the USA is based on both species and origin; permits may restrict the area of release to fall within a natural barrier such as the Continental Divide (USDA-APHIS, 2012). For transgenic insects, the geographical origin of the parent organisms and in some instances the source of transgenes might be considered if reason to suspect species diversification exists.

In an unrelated regulation for setting the level of inspection of incoming agricultural trade, the European Commission (EC) has adopted a formula with mobility of the insect, at its most mobile life stage, as one parameter relating to the risk of spread from hypothetical entry of an infested product (EC, 2004); other factors relate to the amount of data available on actual interceptions. This suggests that a quantification of the mobility factor is feasible in risk assessment as well.

Insect mobility might also be usefully characterized in terms of the species' inherent or biological dispersal ability, together with the possibility of human, wind or other vector-assisted dispersal. Human transport routes may link local populations and provide corridors along which transgenic insects can survive and be transported, and wind-assisted dispersal may transport insects many hundreds of kilometres and across national boundaries (Eagles *et al.*, 2013; Huestis *et al.*, 2019; Epopa *et al.*, 2020).

Lessons about the influence of humans on the spread and dispersal of insects might be learned from outbreaks of plant pests caused by amateur collectors who raise insects in unregulated conditions and, at times, release without permits.

- ability to diapause in unfavourable conditions and revive when conditions improve; and
- possibility of exchanging symbionts, including heritably.

As with other GMOs, important factors frequently cited as potential risk factors associated with transgenic insects include: (i) the nature of the inserted gene itself (source, placement, stability, etc.) and the methodology for modification; (ii) any impact on phenotype or behaviour of the insect that is modified; and (iii) the possible effect of these changes on human health (e.g. vector competence or biting rate), the environment (e.g. non-target organisms) or on the relevant activity, such as crop production or vector control (FAO/IAEA, 2006). These may be direct effects or secondary, for example through gene flow. The assessment in this step is also frequently performed as part of the application process, when seeking a permit to release (Fig. 28.2).

Particular traits are also commonly assumed to be associated with higher risk, as illustrated in Table 28.1. However, it is imperative to substantiate these assumptions with research over time, if not prior to confined release. If information cannot be obtained from confined release, then monitoring in an open field release may provide the necessary data. The endpoints and objectives of such monitoring should be agreed in detail in advance.

Another approach is to build credible scenarios to predict possible, or even worst-case, outcomes from introduced traits (Interdepartmental Liaison Group on Risk Assessment, 2013). When this is done due to insufficient data, the assumptions should be reviewed with each new piece of evidence until each scenario is better quantified in terms of probability and impact.

Some of the characteristics to consider when choosing a site for confined release are outlined in the plant health guidance (NAPPO, 2007), including:

- proximity to populations of the same species as the transgenic arthropod and closely related species;
- proximity to sensitive or protected ecological areas;

- presence of susceptible hosts;
- presence of non-target organisms, beneficial arthropods and endangered or threatened species in the confined field release site (this should take into account the seasonal presence of these organisms, particularly at times of migration and mating); and
- presence of aboriginal populations of the arthropod or closely related species that may be centres of genetic diversity.

Similar considerations should be made for pilot field releases. However, for programmatic use additional considerations may be required.

For transgenic insects/transgenes designed to persist and/or spread genetic material, other considerations will be the similarity of the gene insertion site in other non-target insects, the stability of the insertion and the possible consequences if transfer does occur. In these scenarios, even without scientific evidence of any probability, there may be reason to first perform well-controlled, multi-generation laboratory experiments to eliminate the possibility of creation or transfer of insecticide resistance and acquired ability to vector additional diseases, due to the extreme consequences that could occur. The phased studies, explained above and in Benedict *et al*. (2018a), and use of any methods for mitigating or stopping spread of a gene drive GMO should be used in case of unanticipated outcomes in the field (Benedict *et al*., 2018b).

Such studies and contingency planning are best done with peer review by a group of experts representing all relevant scientific subjects, but who also are cognizant of the limitations of budgets, detection levels and other feasibility factors so that the desire to address concerns does not prevent progress for an intervention that has a high probability of societal benefit.

28.3.3 Risk calculation and characterization

Most national biosafety regulations are agnostic to the methods used in the risk calculation

Table 28.1. Examples of introduced traits and their anticipated relationship to risk

Example characteristics	Expected to have increasing risk		
	→		
Fecundity	Fully sterile	High percentage sterile	Same as target population	Greater than average reproduction rate
Mating competitiveness	Not compatible with native population	Mating compatibility less than native population	Same as target population	Preferred mate and/or able to mate with other species or molecular forms
Dispersal	Non-flying and not transported through other pathways	Disperses in immediate area (e.g., field or house)	Disperses in local area (e.g., village, river basin)	Wide ranging naturally or through other pathways
Lifespan	Lethal trait arises, larval or pupal phase	Early adult mortality	Same as target population	Extended beyond average
Genetic insertion	Demonstrated to be stable over multiple generations in laboratory conditions	Apparently stable over multiple generations in small confined space	Able to transfer to other species under forced laboratory conditions but not in studies mimicking natural conditions	Able to insert into other species in confined conditions, or after open release
Transfer mechanism	Novel genetic material not transferable	Novel genetic material may be transferred in small percentage of cases	Novel genetic material will transfer to approximately half the population through mating	Novel genetic material will transfer in greater than Mendelian percentage (gene drive)

steps, allowing for both qualitative and quantitative approaches (Hayes *et al*., 2013). For gene drive-modified organisms, however, the National Academy of Sciences, Engineering and Medicine (NASEM, 2016) recommends quantitative, probabilistic risk assessments, supported by modelling of off-target and non-target effects from the genome level through to the ecosystem level. Hayes *et al*. (2013) and Finkel *et al*. (2018) expressed similar sentiments in the context of genetically modified fish and synthetic biology, respectively.

Risk calculations can be broadly categorized into two types: qualitative and probabilistic (or quantitative). To date, the majority of risk assessments for the field releases of transgenic insects have been qualitative. The risk analyses used to authorize the relatively small-scale field trial of the GM diamondback moth (*Plutella xylostella*) strain OX4319L (USDA, 2016a), and for the genetically engineered *Aedes aegypti* strain OX513A (USDA, 2016b) and the much larger open field release of this strain of mosquitoes in Brazil (available at: https://bch.cbd.int/database/record.shtml?documentid=105833, accessed 28 October 2021) (see also De Andrade *et al*., 2016), and the most recent release of genetically engineered *Aedes aegypti* strain OX5034 in the Florida Keys (USEPA, 2019, 2020) have all been qualitative.

These qualitative assessments will often cite the (quantitative) results of experiments and field observations relevant to the risk assessment endpoints as evidence for the ordinal (negligible, low, medium, high, etc.) risk predictions that they make. The extent to which this evidence reduces, or better characterizes, the uncertainty associated with the endpoint, however, cannot be accurately represented with qualitative methods. This facet of the risk assessment therefore remains open to interpretation and confounded with the inherent (linguistic) uncertainty associated with qualitative risk predictions.

The risk assessment performed for the small-scale field release of genetically engineered Dominant Sterile Male (DSM) mosquitoes in Burkina Faso (Hayes *et al*., 2015, 2018b; Hosack *et al*., 2021) uses probability theory to represent uncertainty in key risk

assessment parameters and Bayes theorem to amend the uncertainty in light of empirical observations and experimental results. This approach avoids the ambiguity associated with ordinal risk estimates and could in theory provide the basis of a broader comparative assessment of alternative solutions, but it is more resource intensive.

As noted previously, however, the risks associated with the release of transgenic insects are rarely characterized relative to the risk associated with alternative or current solutions for the same problem. In the context of gene drive-modified mosquitoes, James *et al*. (2018) recommended as a safety standard that the release of the transgenic mosquitoes should 'do no more harm to human health than wild-type mosquitoes of the same genetic background and no more harm to the ecosystem than other conventional vector control interventions'. Hence any transgenic solution must demonstrate a net benefit over the status quo or the 'do nothing new' option.

For some endpoints, such as an increase in insecticide resistance or a change in vectorial capacity (Hosack *et al*., 2021), this characterization of risk can be achieved in a transparent fashion. For others, it is much more difficult to do this because the analysis and/or data collection needed to assess the impacts and risks associated with conventional technologies are currently unavailable; see for example the discussion on niche replacement in James *et al*. (2020).

28.3.4 Risk management, communication, acceptability and monitoring

As noted previously, risk assessments for transgenic insects are often qualitative, typically citing a body of evidence to support an ordinal risk prediction. In this paradigm, decisions about the acceptability of risk, and the extent to which risk management measures reduce risk, are based on the interpretation and judgement of the relevant biosafety authority.

The acceptability of a risk judgement amongst the wider community will likely be

determined, at least in part, by how successfully the risk assessment process is communicated. Most modern risk assessment frameworks emphasize that communication with relevant stakeholders and potentially affected communities should occur at all steps in the risk analysis process and should entail more than the simple provision of information. For example, the framework of the Office of the Gene Regulator in Australia (OGTR, 2013) defines risk communication as a 'continual and iterative process to provide, share or obtain information' that is integral to all steps in the risk analysis process.

Monitoring outcomes is the final and arguably most important step of any risk analysis process. The Secretariat of the Convention on Biological Diversity (SCBD, 2016) provided high-level guidance on how to develop and implement monitoring plans for living modified organisms released into the environment.

Observing outcomes and comparing these to predictions are fundamental to any scientific risk assessment. Bayesian inference methods provide a well-formalized way of incorporating observed outcomes into risk predictions, in a way that coherently represents the uncertainty in the prior risk predictions and any subsequent observations, and for this reason they are well suited to scientific assessments of risk, particularly in instances where there is little prior empirical evidence. As noted above, however, probabilistic (Bayesian) approaches are not routinely applied when assessing the risks of transgenic insects.

An adequate assessment must also recognize any limitations inherent to the monitoring and regulatory system available at the site. An early review of the international code for release of biological control agents (FAO, 2017b) showed that few countries were conducting the post-release monitoring as prescribed, at that time (Kairo et al., 2003). This lack of oversight itself forms an additional risk factor. (The review also revealed the value of clear guidance on the roles of each party and the need for a highly engaged leadership to ensure it is taken into account.) It has long been recognized that proposing management measures that are not feasible in the local context invalidates the plan (World Bank, 1999).

28.4 Special Aspects of Risk for Gene Drive Modified Insects

The extent to which gene drive-modified insects (GDMIs) present novel, as opposed to enhanced, hazards and risks is a matter of considerable debate. In our opinion, the risk assessment endpoints identified for GDMIs to date, and the associated pathways to harm, do not point to the existence of *de novo* hazards or risks. GDMIs will, however, present enhanced risk assessment challenges, the magnitude of which will vary depending on the type of gene drive and the circumstances in which it is used.

Devos *et al*. (2021) suggested that GDMIs will display one or more of the following three intentionally novel characteristics: (i) a preferential rate of inheritance; (ii) a larger spatial and temporal scale of spread; and (iii) the possibility of population modification strategies. By way of contrast, the authors did not consider intentional population suppression strategies as novel, because SIT and classical biological control had been used previously to achieve local and area-wide suppression, although one might again argue that the spatio-temporal magnitude of suppression, if not larger, is easier to achieve.

Santos (2020) also identified the potential scale of spread and persistence – along with uncertainty about costs, benefits and harms, and successful community engagement – as the key challenges when evaluating the potential benefits and risks of GDMIs. These challenges are of course intricately linked, because variability in risk factors increases with the spatial and temporal scope of a risk assessment, and successful community engagement becomes more difficult as the size and diversity of the potentially affected community increases.

In its recent report the Ad Hoc Technical Expert Group on risk assessment (AHTEG, 2020), established by the Conference of the Parties serving as the Meeting of the Parties to the Cartagena Protocol on Biosafety,

identified a much longer set of risk assessment challenges, categorized in groups relevant to the gene drive system, the target organism, the receiving environment, risk methods, data collection and risk management and monitoring.

Many of the specific challenges are again linked to, driven by, or exacerbated by the potential large spatial and temporal scale that GDMI risk assessments may have to contend with, for example depending on the type of gene drive system that is used. In this sense, the large spatial and temporal scope can be viewed as a central issue from which many specific challenges originate, such as the difficulty of obtaining data to accurately characterize the biophysical characteristics of the receiving environment and the logistical challenges associated with post-release monitoring.

The central role that the spatial and temporal scale of the assessment plays in many of the challenges associated with risk assessments for field releases of GDMIs emphasizes the importance of phased development and release strategies that enable the spatio-temporal footprint of the field release to be controlled and gradually increased.

28.5   Interactions and Cumulative Risk

As already suggested, it is difficult to predict all interactions that may affect risk after field release. Furthermore, the same receiving environment may undergo changes from the time of the original evaluation and risk analysis. It is not realistic to require the original developer, research team or project which introduces the product for public sector uptake to be responsible for monitoring and observing all of these future interactions in order to revise the original assessments. Even a commercial entity with long-term presence in the market does not control and may be unaware of the multiple changes to the receiving environment which could alter the behaviour or performance of the living product.

Therefore, the national or local government will be required to consider these issues when approving a release. One way to facilitate efficient review of decisions is to prepare a table of possible trigger events or conditions for changes in the risk. This could be related to: climate (e.g., drought, floods, increasing average temperatures); species complex (e.g., introduction of another species, loss of species diversity, shift in keystone species); demography (e.g., increase in density of human population, change in movement of people); pest control practices (e.g., overuse of a particular insecticide, substitution of pesticides with biocontrol agents); and so forth. By identifying possible triggers, with credible mechanisms for affecting the transgenic insect, the burden on the public sector to review their decisions is alleviated.

The key elements of an Integrated Confinement System, albeit initially related to crops, have been identified (NRC, 2004) as:

- commitment by top management;
- establishment of written plans to be implemented, including those for documentation, monitoring, and remediation;
- training of employees;
- dedication of permanent staff to maintain continuity;
- use of standard operating procedures and good management practices;
- periodic audits by an independent entity;
- periodic internal review and adaptive management; and
- reporting to an appropriate regulatory body.

It would seem that, in addition to environmental factors, these elements are key throughout pilot and open field releases and even after the transgenic insect has become part of an ongoing programmatic intervention. People will always be one of the most important factors in all work with transgenics – both as sources of risk and as resources for the prediction and management of risk.

28.6   Documentation of Risk Analysis

Documentation of assumptions, uncertainty, endpoints and data or data sources is critical to get the most from a risk analysis. Adequate documentation extends the value of the

analysis from decision support at one point in time, to a range of purposes over time: (i) a record that can be taken up by others to confirm or adjust risk assumptions as new information is obtained; (ii) a communication tool to, for example, inform the public how their concerns were taken into account or to interact with regulators and politicians on cross-border issues; (iii) as a transparent record of the process to provide legitimacy of permit decisions if questioned over time; and (iv) to support development of risk management and standard operating procedures.

Final conclusive reports are important, but risk analysis may be carried out at several steps in the development of a product, as shown in Fig. 28.3. A risk analysis may be required as a part of each of the documents noted: an import permit application, a field release permit application, an ethics review, an environmental impact assessment, an impact evaluation and a monitoring plan. Similarly, these studies may contribute sections

to a comprehensive risk analysis. The exact nature of documentation will be set by the national or, at times, local regulatory requirements.

28.7 Social and Political Aspects of Risk

Article 26 of the Cartagena Protocol states that Parties '*may take into account* socioeconomic considerations' and, in Article 23, that they '*shall promote and facilitate* public ... participation ... [and] consult the public' [emphasis added]. De Andrade *et al.* (2012) warned that the former may cause conflicts with rights and responsibilities for other international agreements. This level of public participation is often new for regulators. Mumford and Carrasco (Chapter 26, this volume) discuss precedent and ways to consider economic factors in a large transgenic insect release programme.
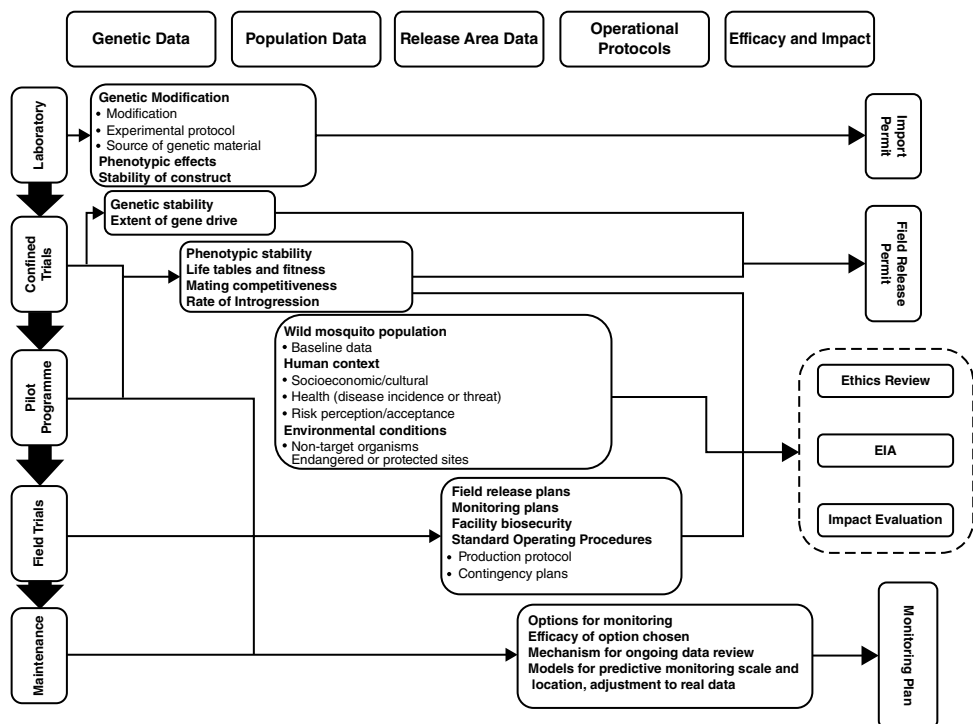
Fig. 28.3. Phases of technology development, data types and requirements and resulting documentation for deployment of a GM mosquito. (Source: Knight *et al.,* 2010.)

Macer (2003) was one of the first to document key social and ethical issues in regard to release of GM mosquitoes. Researchers themselves have recognized the need for broader agreement on social, ethical and legal aspects of moving from laboratory to field applications of transgenic insects (WHO, 2010). Some advances along this line have been made in confined and pilot research-and-release site selection and community engagement (Lavery *et al.*, 2008, 2010; Hartley *et al.*, 2019; Pare Toe *et al.*, 2021). Thizy *et al.* (Chapter 24, this volume) discuss more recent initiatives to include such issues as a more central tenet to decision making. An important question for this chapter is how much of this process should be taking part in the risk assessment phase, versus during the analysis for the risk management and communication phases, or through other mechanisms entirely.

The Hazard Identification step (which can be separated from the assessment phase) is an important point at which to obtain broader input, so that valid concerns will not be left out of the analysis process. The Centre for Environmental Policy at Imperial College London has developed a series of opinion elicitation and graphic representational tools for policy formulation based on risk analysis with high uncertainty or lack of data (e.g., Leach and Mumford, 2008; Mumford *et al.*, 2010).

Presenting opinion as a distribution and eliciting certainty about opinion as an additional dimension (e.g., Holt *et al.*, 2012, 2014) has allowed for the identification of clusters and trends in concerns, which may inform Hazard Identification. (Concerns that have no scientific evidence may be included in this type of exercise, but these are then identified as hazards in terms of the possible impact from people believing in the concern rather than as a scientifically supported hazard in and of itself.) This method seems to work best with expert groups who are at least somewhat familiar with the topic and able to express opinions in quantitative scales and evaluate their own certainty.

The Grand Challenges in Global Health-supported project for release of *Wolbachia*-infected mosquitoes has used a hierarchical modelling system that can express relationships of cause and effect, structuring this as a Bayesian network, in order to capture public input in the same risk assessment-style study as the biological, epidemiological and programmatic concerns identified through focus groups and surveys (Murphy *et al.*, 2010). This method of representing hazards or concerns and their probability (initially as predicted by the stakeholder raising the concern) has been taken up by Vietnamese project implementers in a 3-month process of elicitation from expert groups, workshop and final analysis (Eliminate Dengue Vietnam, 2011).

Risk assessment case studies were developed for transgenic tephritid fruit flies, pink bollworm and *Ae. aegypti* mosquitoes at a UNDP-sponsored workshop in Malaysia on risk assessment of transgenic insects (Beech *et al.*, 2009). Hazards were identified with a time-frame, mechanisms or pathways to the hazard, and estimate of the likelihood. It was found that most experts outside the field of risk do not readily distinguish between hazard, consequence, risk and uncertainty. This study concluded that a risk assessment is not generally familiar to even technical audiences and that it may not be the appropriate tool for capturing ethical, social and cultural concerns. A similar methodology has been used more recently in Africa (Roberts *et al.,* 2017).

For frameworks that distinguish a separate, initial phase of Problem Formulation, this is another opportunity to incorporate public opinion, social and cultural issues and political priorities. Some hazards, such as pathogens, are already classified through international systems that are employed by most governments. Risk acceptability for transgenic insects is not so clearly described or known. The Australian Office of the Gene Technology Regulator begins its decision process with definition of the risk context (OGTR, 2013). This includes the development of a 'Consequence Assessment Criteria Matrix', which uses descriptors that could be tested against public acceptance of risk and concerns.

An illustrative Consequence Matrix for a serious agricultural pest is shown in Table 28.2. This would allow stakeholders to

Table 28.2. Illustrative Consequence Assessment Criteria Matrix for use in determining community acceptance of a novel plant protection intervention (such as a GM crop).

Level of harm	Challenge	Impact
Marginal	New plant pest or disease incursion, being addressed with existing control measures	Minimal impact on market prices, consumer availability or quality of food items
Minor	New plant pest or disease established and affecting entire production sector for a preferred food item	Some food items not available or too high cost but can be substituted with others
Intermediate	New pest or disease established, not responding to available measures, and affecting entire production sector for a core food item	Core food items only available at significantly higher prices
Major	Serious pest or disease established and spreading through the region. Control measures not effective or causing other damage and/or are not feasible for significant portions of the population	Country-wide impact on availability of core food items, significant increase in prices

express their concerns and acceptance or aversion to risk in terms of the context of the problem or challenge. This type of matrix may be developed in advance of a risk assessment, so as to provide input on risk acceptance and community views.

Analysis of the application of the Cartagena Protocol (McLean *et al.*, 2012) suggested that the precautionary principle has been used to restrict decisions to environmental criteria rather than all of the criteria, or even the top-priority criteria, at hand. The balance of benefit and risk allows for scientific, social and economic development rather than single-sector authority over national decisions (Nuffield Council on Bioethics, 2004).

Much remains to be developed in terms of inclusion of social and political factors in a decision regarding use of transgenic insects. Certainly, the degree to which public good needs to be demonstrated or agreed and the concept of community consent require further study. Although hazards and concerns may be identified by the public, it would seem that the step of estimating risk should be handled by risk assessors rather than requiring such estimates from the general public.

28.8 Conclusions

Assessing risk of transgenic insects can be informed by long-established practices in risk assessment as applied in other fields, particularly for release of living organisms such as biocontrol agents or to some extent for GM microorganisms that are self-sustaining. (For laboratory research and contained studies, existing guidance already encompasses most risks associated with transgenic insects.)

The same concepts – including use of risk analysis – are the basis for virtually all biosafety resource documents, for example from OECD, International Organization for Biological Control (IOBC) and other multilateral or intergovernmental treaties mentioned above. Initially, guidance was focused on general principles and GM plants and seed. Guidance relating to GM animals to identify unique aspects of risk assessment and analysis for animals is evolving, although focusing on livestock rather than insects. Official regional and national guidance on GM insects is slowly catching up with the initiatives of the scientific communities and multilateral agencies. This includes guidance from the Cartagena Protocol, reporting on issues in risk assessment overall and of Living Modified Mosquitoes (AHTEG, 2010, 2012) which is still somewhat imprecise in concepts. The European guidelines (EFSA, 2013) do not have regulatory status but were developed with the characteristics of GM insects in mind.

A number of contributions from academic, research, business and development sectors propose greater detail on risk features of

transgenic insects and useful methods for quantification of risk. Voluntary 'precaution' has been a hallmark of the research community since the first studies with GM insects. Some factors are more specific to assessing risks of transgenic insects. In these cases, national biosafety regulations may require adjustment to be fit for purpose. Harmonization of data requirements would benefit commercial and public research, as discussed by Beech *et al.* (Chapter 25, this volume). In addition to biological risks, it is important to consider the role and impact of novel technologies on the current insect control programmes in agriculture or public health. Vector control poses additional concerns in relation to disease dynamics.

Multilateral guidance documents provide a window into shared ideas and values, whilst some national or regional regulatory regimes retain variations on concepts so that harmonization is unlikely. The vast majority of developing countries are Parties to the Cartagena Protocol and have established biosafety frameworks under the auspices of the CBD and the Global Environmental Facility and are relying on the Cartagena Protocol for capacity and guidance. Thus, environmental criteria may continue to dominate what should be a cross-sectorial decision. Capacity in risk assessment will likely develop more quickly if linked with actual uses of biotechnology for commercial or public good.

Most would agree that the 'regulatory polarization', attributed by Bernauer (2003) and others to differences in public opinion and risk acceptance, has caused barriers to beneficial use of biotechnology and wasted valuable resources for governments already facing food security and public health challenges. Yet a fresh look at how to incorporate some of these factors comprising risk acceptance could possibly advance the discussion on harmonization of methodology. A positive result of the higher standards imposed on biotechnology at the beginning of harmonized regulation is that assessment of novel organisms can provide a catalyst for thought on issues previously not addressed for other agriculture or health interventions (Macer, 2003).

Societies should guard against requiring too much of risk analysis, however. Political pressures on an essentially technical and scientific exercise can weaken the process. Legitimate issues related to social, political, environmental and other priorities can be taken into account during selection of the risk management options and policy formulation, and through deeper stakeholder involvement (Thizy *et al*., Chapter 24, this volume). Risk analysis can support decision making by providing iterative biosafety analysis and operational planning for field use of transgenic insects. It can also provide a mechanism for public engagement. More rigorous and inclusive methods will be required, however, if it is to legitimize the overall regulatory process (NRC, 2002), otherwise the methodology will likely fall short of expectations.

Acknowledgements

References

ACME (American Committee of Medical Entomology) (2019) Arthropod Containment Guidelines, version 3.2. *Vector-Borne and Zoonotic Diseases* 3(2), 57–98.

ACME/ASTMH (2021) Containment Practices for Arthropods Modified with Engineered Transgenes Capable of Gene Drive. Addendum 1 to the Arthropod Containment Guidelines, Version 3.2. *Vector-Borne and Zoonotic Diseases* 22(1), 3–17. Published online (18 October 2021) ahead of print (13 January 2022). doi: 10.1089/vbz.2021.0035

AHTEG (Ad Hoc Technical Expert Group) (2010) Living modified mosquitoes. Section C in: *Final report of the Ad Hoc Technical Expert Group on Risk Assessment and Risk Management under the Cartagena Protocol on Biosafety*. UNEP/CBD/BS/COP-MOP/5/INF/15, 3 August 2010. Commissioned by:

Conference of the Parties to the Convention on Biological Diversity serving as the Meeting of the Parties to the Cartagena Protocol on Biosafety, Fifth Meeting, Nagoya, Japan, 11–15 October 2010. Available at: https://bch.cbd.int/onlineconferences/ahteg_past_activities.shtml (accessed October 2021)

AHTEG (2012) *Final report of the Ad Hoc Technical Expert Group on Risk Assessment and Risk Management under the Cartagena Protocol on Biosafety*. UNEP/CBD/BS/COP-MOP/INF/10, 21 June 2012. Commissioned by: Conference of the Parties to the Convention on Biological Diversity serving as the Meeting of the Parties to the Cartagena Protocol on Biosafety, Sixth Meeting, Hyderabad, India, 1–5 October 2012. Available at: https://bch.cbd.int/onlineconferences/ahteg_past_activities.shtml http://bch.cbd.int/ (accessed October 2021).

AHTEG (2020) *Risk Assessment and Risk Management*. CBD Subsidiary Body of Scientific, Technical and Technological Advice (SBSTTA). Twenty-fourth meeting Quebec, Canada, 2–7 November 2020. Item 5 of the provisional agenda. Available at: https://www.cbd.int/doc/c/c0ed/d93b/438ae23fd9e6600b-f15205b1/sbstta-24-05-en.docx (accessed October 2021).

Akbari, O.S., Bellen, H.J., Bier, E., Bullock, S.L., Burt, A. *et al.* (2015) Safeguarding gene drive experiments in the laboratory. *Science* 349 (6251), 927–929.

Annas, G.J., Beisel, C.L., Clement, K., Crisanti, A., Francis, S. *et al.* (2021) A Code of Ethics for Gene Drive Research. *The CRISPR Journal* 4(1), 19–24. doi.org/10.1089/crispr.2020.0096

Beaty, B., Prager, D., James, A., Jacobs-Lorena M., Miller, L. *et al.* (2009) From Tucson to genomics and transgenics: the vector biology network and the emergence of modern vector biology. *PLoS Neglected Tropical Diseases* 3(3), e343. doi: 10.1371/journal.pntd.0000343

Beech, C., Nagaraju, N. Vasan, S.S. and Rose, R.I. (2009) Risk analysis of a hypothetical open field release of a self-limiting transgenic *Aedes aegypti* mosquito strain to combat dengue. *Asia-Pacific Journal of Molecular Biology and Biotechnology* 17(3), 97–108.

Benedict, M.Q and Robinson, A.S. (2003) The first releases of transgenic mosquitoes: an argument for the sterile insect technique. *Trends in Parasitology* 19(8), 349–355.

Benedict, M., D'Abbs, P., Dobson, S., Gottlieb, M., Harrington, L. *et al.* (2008) Guidance for contained field trials of vector mosquitoes engineered to contain a gene drive system: Recommendations of a scientific working group. *Vector-Borne and Zoonotic Diseases* 8, 127–164.

Benedict, M.Q., Burt, A., Capurro, M.L., De Barro, P., Handler, A.M. *et al.* (2018a) Recommendations for laboratory containment and management of gene drive systems in arthropods. *Vector-Borne and Zoonotic Diseases*, 18 (1). doi.org/10.1089/vbz.2017.2121

Benedict, M.Q., Charlwood, J.D., Harrington, L.C., Lounibos, L.P., Reisen, W.K. and Tabachnick, W.J. (2018b) Guidance for evaluating the safety of experimental releases of mosquitoes, emphasizing mark-release-recapture techniques. *Vector-Borne and Zoonotic Diseases* 18(1). doi.org/10.1089/vbz.2017.2152

Bernaeur, T. (2003) *Genes, Trade, and Regulation: The Seeds of Conflict in Food Biotechnology*. Princeton University Press, Princeton, New Jersey.

Brown, D.M., Alphey, L.S., McKemey, A., Beech, C. and Lames, A.A. (2014) Criteria for identifying and evaluating candidate sites for open-field trials of genetically engineered mosquitoes. *Vector-Borne and Zoonotic Diseases* 14(4), 291–299. doi: 10.1089/vbz.2013.1364

Bulkeley, H., Jordan, A., Perkins, R. and Selin, H. (2013) Governing sustainability: Rio+20 and the road beyond. *Environment and Planning C: Government and Policy* 31, 958–970.

Burt, A. (2014) Heritable strategies for controlling insect vector diseases. *Philosophical Transactions of the Royal Society B* 369 (20130432).

Carvalho, D.O., McKemey, A.R., Garziera, L., Lacroix, R., Donnelly, C.A. *et al.* (2015) Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. *PLoS Neglected Tropical Diseases* 9(7).

Concha, C., Palavesam, A., Guerrero, F.D., Sagel, A., Li, F. *et al.* (2016) A transgenic male-only strain of the New World screwworm for an improved control program using the sterile insect technique. *BMC Biology* 14, 72. doi: 10.1186/s 12915-016-0296-8

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N. *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823.

Connolly, J.B., Mumford, J.D., Fuchs, S., Turner, G., Beech, C. *et al.* (2021) Systematic identification of plausible pathways to potential harm via problem formulation for investigational releases of a population suppression gene drive to control the human malaria vector *Anopheles gambiae* in West Africa. *Malaria Journal* 20, 170. doi: 10.1186/s12936-021-03674-6

De Andrade, P.P. Parrott, W. and M.M., Roca (eds) (2012) *Guía para la Evaluación de Riesgo Ambiental de Organismos Genéticamente Modificados*. International Life Sciences Institute, Sâo Paulo, Brazil.

De Andrade, P.P., Lima Aragao, F.J., Colli, W., Dellagostin, O.A., Finardi-Filho, F. *et al.* (2016) Use of transgenic *Aedes aegypti* in Brazil: risk perception and assessment. *Bulletin of the World Health Organization* 94, 766–771.

Devos, Y., Craig, W., Devlin, R.H., Ippolito, A., Leggatt, R.A. *et al.* (2019) Using problem formulation for fit-for-purpose pre-market environmental risk assessments of regulated stressors. *EFSA Journal* 17(S1).

Devos, Y., Mumford, J.D., Bonsall, M.B., Camargo, A.M., Firbank, L.G. *et al.* (2021) Potential use of gene drive modified insects against disease vectors, agricultural pests and invasive species poses new challenges for risk assessment. *Critical Reviews in Biotechnology* 42(2). doi: 10.1080/07388551.2021.1933891

Eagles, D., Walker, P.J., Zalucki, M.P. and Durr, P.A. (2013) Modelling spatio-temporal patterns of long-distance Culicoides dispersal into northern Australia. *Preventive Veterinary Medicine* 110, 312–322.

EC (2004) Commission Regulation (EC) No. 1756/2004 of 11 October 2004 specifying the detailed conditions for the evidence required and the criteria for the type and level of the reduction of the plant health checks of certain plants, plant products or other objects listed in Part B of Annex V to Council Directive 2000/29/EC. *Official Journal of the European Union*, 12 October 2004, pp. L313/6–L313/9.

EFSA (European Food Safety Authority) (2013) Guidance on the environmental risk assessment of genetically modified animals. EFSA Panel on Genetically Modified Organisms (GMO). *EFSA Journal* 11(5), 3200.

EFSA (European Food Safety Authority) (2020) *Outcome of a public consultation on the draft adequacy and sufficiency evaluation of existing EFSA guidelines for the molecular characterisation, environmental risk assessment and post-market environmental monitoring of genetically modified insects containing engineered gene drives*. EFSA Supporting publication 2020: EN-1939. doi: 10.2903/sp.efsa.2020.EN-1939

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms) (2020) Scientific Opinion on the adequacy and sufficiency evaluation of existing EFSA guidelines for the molecular characterisation, environmental risk assessment and post-market environmental monitoring of genetically modified insects containing engineered gene drives. *EFSA Journal* 18(11), 6297. doi: 10.2903/j.efsa.2020.6297

Eliminate Dengue Vietnam (2011) Risk assessment of the pilot release of *Aedes aegypti* mosquitoes containing *Wolbachia*. [English version.] Available at: www.eliminatedengue.com/library/publication/document//july_2011_ra_report_eng.pdf (accessed October 2021).

Enkerlin, W. and Zavala-López, J.L. (2017) *Guidance for Packing, Shipping, Holding and Release of Sterile Flies in Area-wide Fruit Fly Control Programmes*. FAO Plant Production and Protection paper 190. FAO, Rome.

Epopa, P.S, Millogo, A.A., Collins, C.M., North, A.R., Benedict, M.Q. *et al.* (2020) *Anopheles gambiae* (s.l.) is found where few are looking: assessing mosquito diversity and density outside inhabited areas using diverse sampling methods. *Parasites & Vectors* 13, 516.

EPPO (2011) *Decision-support Scheme for Quarantine Pests*. Guidelines on Pest Risk Analysis PM 5/3 (5). European and Mediterranean Plant Protection Organization, Paris. Available at: https://www.eppo.int/RESOURCES/eppo_standards/pm5_pra (accessed October 2021)

FAO (2011) *Biosafety Resource Book*. Food and Agriculture Organization, Rome.

FAO (2017a) ISPM 11. *Pest Risk Analysis for Quarantine Pests including Analysis of Environmental Risks and Living Modified Organisms*. Endorsed 2013. [Current adopted versions of ISPMs are available for download on www.ippc.int.] International Plant Protection Convention (IPPC), Rome.

FAO (2017b) ISPM 3. *Guidelines for the Export, Shipment, Import and Release of Biological Control Agents and other Beneficial Organisms*. Endorsed 2005. [Current adopted versions of ISPMs are available for download on www.ippc.int.] International Plant Protection Convention (IPPC), Rome.

FAO (2019) ISPM 2. *Framework for Pest Risk Analysis*. Endorsed 2007. [Current adopted versions of ISPMs are available for download on www.ippc.int.] International Plant Protection Convention (IPPC), Rome.

FAO (2021) Appendix 1: *Terminology of the Convention on Biological Diversity in relation to the Glossary of Phytosanitary Terms*. Endorsed 2001 revised 2012. [Current adopted versions of ISPMs are available for download on www.ippc.int.] In: *ISPM 5. Glossary of Phytosanitary Terms*. International Plant Protection Convention (IPPC), Rome.

FAO/IAEA (2006) *Status and Risk Assessment of the Use of Transgenic Arthropods in Plant Protection*. Report of the Joint FAO/IAEA Division and Secretariat of the IPPC, FAO Consultants Meeting, Rome, Italy, 8–12 April. IAEA-TECDOC-1483. FAO/IAEA, Vienna.

Finkel, A.M., Trump, B.D., Bowman, D. and Maynard, A. (2018) A 'solution-focused' comparative risk assessment of conventional and synthetic biology approaches to control mosquitoes carrying the dengue fever virus. *Environment Systems and Decisions* 38, 177–197. doi: 10.1007/s10669-018-9688-3

Government of Malaysia (2010) *Risk assessment report of the Genetic Modification Advisory Committee (GMAC) for an application to conduct a limited mark–release–recapture of Aedes aegypti (L.) wild type and OX513A strains*. Available at: http://bch.cbd.int/database/attachment/?id=10793 (accessed October 2021)

Government of Malaysia (2013a) *Living modified organism identity imported into Malaysia, 28 April 2013. Dominant lethal Aedes aegypti mosquito*. Available at: http://bch.cbd.int/database/record.shtml?-documentid=101474 (accessed October 2021)

Government of Malaysia (2013b) *Notification of importation of* Aedes aegypti *into Malaysia, 3 April 2013*. Available at: http://bch.cbd.int/database/record.shtml?documentid=101481 (accessed October 2021)

Handler, A. (2002) Use of the piggyBac transposon for germ-line transformation of insects. *Insect Biochemistry and Molecular Biology* 32, 1211–1220.

Hartley, S., Thizy, D., Ledingham, K., Coulibaly, M., Diabaté, A. *et al.* (2019) Knowledge engagement in gene drive research for malaria control. *PLoS Neglected Tropical Diseases* 13, e0007233. doi: 10.1371/journal.pntd.0007233

Hayes, K.R. (2004) *Robust Methodologies for Ecological Risk Assessment. Best Practice and Current Practice in Ecological Risk Assessment for Genetically Modified Organisms*. CSIRO Division of Marine Research, Hobart, Tasmania, Australia.

Hayes, K.R., Kapuscinski, A.R., Dana, G., Li, S. and Devlin, R.H. (2007) Introduction to environmental risk assessment for transgenic fish. In: Kapuscinski, A.R., Hayes, K.R., Li, S., Dana, G., Hallerman, E.M. and Schei, P.J. (eds) *Environmental Risk Assessment of Genetically Modified Organisms. Volume 3: Methodologies for Transgenic Fish*. CABI, Wallingford, UK, pp. 1–28.

Hayes, K.R., Leung, B., Thresher, R., Dambacher, J.M. and Hosack, G.R. (2013) Meeting the challenge of quantitative risk assessment for genetic control techniques: a framework and some methods applied to the common Carp (*Cyprius carpio*) in Australia. *Biological Invasions* 16, 1273–1288.

Hayes, K.R., Barry, S.C., Beebe, N., Dambacher, J.M., De Barro, P., *et al.* (2015) *Risk Assessment for Controlling Mosquito Vectors with Engineered Nucleases, Sterile Male Construct: Final report*. CSIRO Biosecurity Flagship, Hobart, Australia, 202 pp.

Hayes, K.R., Hosack, G.R., Dana, G.V., Foster, S.D., Ford, J.H. *et al.* (2018a) Identifying and detecting potentially adverse ecological outcomes associated with the release of gene-drive modified organisms. *Journal of Responsible Innovation* 5 (Suppl. 1) S139–S158. doi: 10.1080/23299460.2017.1415585

Hayes, K.R., Hosack, G.R., Ickowicz, A., Foster, S., Peel, D. *et al.* (2018b) *Risk Assessment for Controlling Mosquito Vectors with Engineered Nucleases: Controlled field release for Sterile Male Construct: Risk assessment. Final report*. CSIRO Data61, Hobart, Australia.

Hilbeck, A. and Meier, M. (2006) The guidelines project of the IOBC global WG and risk assessment of transgenic arthropods. In: *Status and Risk Assessment of the Use of Transgenic Arthropods in Plant Protection*. Report of the Joint FAO/IAEA Division and Secretariat of the IPPC, FAO Consultants Meeting, Rome, Italy, 8–12 April. IAEA-TECDOC-1483. FAO/IAEA, Vienna, pp. 107–112.

Holt, J., Leach, A.W., Knight, J.D., Griessinger, D., MacLeod, A. *et al.* (2012) Tools for visualizing and integrating pest risk assessment ratings and uncertainties. *Bulletin OEPP/EPPO Bulletin* 42(1), 35–41.

Holt, J., Leach, A.W., Schrader, G., Petter, F., MacLeod, A. *et al.* (2014) Eliciting and combining decision criteria using a limited palette of utility functions and uncertainty distributions: illustrated by application to pest risk analysis. *Risk Analysis* 34(1), 4–16.

Hosack, G.R., Ickowicz, A. and Hayes, K.R. (2021) Quantifying the risk of vector-borne disease transmission attributable to genetically modified vectors. *Royal Society Open Science* 8, 201525. doi: 10.1098/rsos.201525

Hoy, M.A. (2000) Deploying transgenic arthropods in pest management programs: risks and realities. In: Handler, A. and James, A.A. (eds) *Insect Transgenesis, Methods and Applications*. CRC Press, Boca Raton, Florida, pp. 335–367.

Huestis, D.L., Dao, A., Diallo, M., Sanogo, Z.L., Samake, D. *et al.* (2019) Windborne long-distance migration of malaria mosquitoes in the Sahel. *Nature* 574(7778), 404–408. doi.org/10.1038/s41586-019-1622-4

Hutchison, W.D., Burkness, E.C., Carrillo, M.A., Galvan, T.L., Mitchell, P.D. *et al.* (2006) *Integrated Pest Management: A Risk Management Framework to Improve Decision-making*. University of Minnesota Extension Service, St Paul, Minnesota.

IAEA (International Atomic Energy Agency) (2008) *Model Business Plan for a Sterile Insect Production Facility*. Joint FAO/IAEA Programme: Nuclear Techniques in Food and Agriculture, Vienna.

Interdepartmental Liaison Group on Risk Assessment (2013) *The precautionary principle: policy and application*. Health and Safety Executive, UK. Available at: www.hse.gov.uk/aboutus/meetings/committees/ilgra/ (accessed October 2021)

James, A.A. (2005) Gene drive systems in mosquitoes: rules of the road. *Trends in Parasitology* 21(2), 64–67.

James, S., Collins, F.H., Welkhoff, P.A., Emerson, C.J., Godfray, H.C. *et al.* (2018) Pathway to deployment of gene drive mosquitoes as a potential biocontrol tool for elimination of malaria in sub-Saharan Africa: Recommendations of a scientific working group. *American Journal of Tropical Medicine and Hygiene* 98, 1–49. doi: 10.4269/ajtmh.18-0083

James, S.L., Marshall, J.M., Christophides, G.K., Okumu, F.O. and Nolan, T. (2020) Toward the definition of efficacy and safety criteria for advancing gene drive-modified mosquitoes to field testing. *Vector-Borne and Zoonotic Diseases* 20(4), 237–251. doi: 10.1089/vbz.2019.2606

Johnston, S., Monagle, C., Green, J. and Mackenzie, R. (2008) *Internationally Funded Training in Biotechnology and Biosafety: Is it Bridging the Biotech Divide?* United Nations University Institute of Advanced Studies, Yokohama, Japan.

Kairo, M., Cock, M.W.J. and Quinlan, M.M. (2003) An assessment of the use of the Code of Conduct for the Import and Release of Exotic Biological Control Agents (ISPM No. 3) since its endorsement as an international standard. *Biocontrol News and Information* 24(1), 15N–27N.

Kandul, N.P., Belikoff, E.J., Liu, J., Buchman, A., Li, F. *et al.* (2021) Genetically encoded CRISPR components yield efficient gene editing in the invasive pest *Drosophila suzukii*. *The CRISPR Journal* 2021, 739-751. doi: 10.1089/crispr.2021.0032

Kaplan, S. and Garrick, B.J. (1981) On the quantitative definition of risk. *Risk Analysis* 1(1), 11–27.

Kapuscinski, A.R. (2002) Controversies in designing useful ecological assessments of genetically engineered organisms. In: Letourneau, D. and Burrows, B. (eds) *Genetically Engineered Organisms: Assessing Environmental and Human Health Effects*. CRC Press, Boca Raton, Florida, pp. 385–415

Keese, P. (2008) Risks from GMOs due to horizontal gene transfer. *Environmental Biosafety Research* 7, 123–149.

Knight, J.D., Rhodes, J., McKemey, A., Quinlan, M.M., Beech, C. *et al.* (2010) *Transgenic mosquitoes: best practice guidance. Data requirements for field release and monitoring*. Abstracts of the 11th International Symposium on the Biosafety of Genetically Modified Organisms, Buenos Aires, Argentina, 15–20 November 2010. *Oxford Mills*, Ontario, Canada, International Society for Biosafety Research, p. 186

Knols, B.G.J. and Louis, C. (eds) (2006) *Bridging Laboratory and Field Research for Genetic Control of Disease Vectors*. Wageningen UR Frontis Series, Vol. 11. Springer, Dordrecht, Netherlands.

Kuzma, J. (2019) Procedurally robust risk assessment framework for novel genetically engineered organisms and gene drives. *Regulation & Governance* 15(4), 1144–1165. doi.org/10.1111/rego.12245

Lavery, J., Harrington, L. and Scott, T. (2008) Ethical, social and cultural considerations for site selection for research with genetically modified mosquitoes. *American Journal of Tropical Medicine and Hygiene* 79, 312–318.

Lavery, J., Tinadana, P.O., Scott, T.W., Harrington, L., Ramsey, J. *et al.* (2010) Towards a framework for community engagement in global health research. *Trends in Parasitology* 26(6), 279–283.

Leach, A.W. and Mumford, J.D. (2008) Pesticide environmental accounting: a method for assessing the external costs of individual pesticide applications. *Environmental Pollution* 151, 139–147.

Leppla, N. and De Clercq, P. (2019) History of the International Organization for Biological Control Global Working Group on Mass Rearing and Quality Assurance. *Journal of Insect Science* 19(2), 7, 1–12.

Lindholm, A.K., Dyer, K.A., Firman, R.C., Fishman, L., Forstmeier, W. *et al.* (2016) The ecology and evolutionary dynamics of meiotic drive. *Trends in Ecology and Evolution* 31(4), 314–326.

Macer, D. (2003) *Ethical, Legal and Social Issues of Genetically Modified Disease Vectors in Public Health*. UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), Geneva, Switzerland.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M. *et al.* (2013) RNA-Guided human genome engineering via Cas9. *Science* 339, 823–826.

McLean, M., Foley, M.E. and Pehu, E. (2012) *The Status and Impact of Biosafety Regulation in Developing Economies Since Ratification of the Cartagena Protocol*. Joint Departmental Discussion Paper 3. World Bank, Washington, DC.

Mumford, J.D., Booy, O., Baker, R.H.A., Rees, M., Copp, G.H. *et al.* (2010) Invasive species risk assessment in Great Britain. *Aspects of Applied Biology* 104, 49–54.

Mumford, J.D., Leach, A.W., Benedict, M.Q., Facchinelli, L. and Quinlan, M.M. (2018) Maintaining quality of candidate strains of transgenic mosquitoes for studies in containment facilities in disease endemic countries. *Vector Borne Zoonotic Diseases* 18(1), 31–38. doi:10.1089/vbz.2017.2208

Murphy, B., Jansen, C., Murray, J. and De Barro, P. (2010) *Risk analysis on the Australian release of Aedes aegypti (L.) (Diptera: Culicidae) containing Wolbachia*. CSIRO Report. Available at: https://www.world-mosquitoprogram.org/en/learn/scientific-publications/risk-analysis-australian-release-aedes-aegypti-l-diptera-culicidae (accessed October 2021)

NAPPO (North American Plant Protection Organization) (2007) RSPM No. 27. *Guidelines for Importation and Confined Field Release of Transgenic Arthropods in NAPPO Member Countries*. NAPPO Regional Standards for Phytosanitary Measures (RSPMs). [This standard is now archived.] Secretariat of NAPPO, Ottawa, Canada.

NASEM (National Academy of Sciences, Engineering, and Medicine) (2016) *Gene Drives on the Horizon: Advancing Science, Navigating Uncertainty, and Aligning Research with Public Values*. National Academies Press, Washington, DC. doi: 10.17226/23405

Nelson, K.C., Basiao, Z., Cooper, A.M., Dey, M., Fonticiella, D. *et al.* (2007) Problem Formulation and Options Assessment: 29 Science-guided Deliberation in Environmental Risk Assessment of Transgenic Fish. In: Kapuscinski, A.R., Hayes, K.R., Li, S. and Dana, G. (eds) *ERA for Transgenic Fish*. CAB International, Wallingford, UK.

NIH (2019) *NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules*. April 2019 revision of earlier guidelines. National Institutes of Health, Bethesda, Maryland. Available at: https://osp.od.nih.gov/wp-content/uploads/NIH_Guidelines.pdf (accessed October 2021)

NRC (National Research Council) (1996) *Understanding Risk: Informing Decisions in a Democratic Society*. National Academy Press, Washington DC.

NRC (National Research Council) (2002) *Environmental Effects of Transgenic Plants: the Scope and Adequacy of Regulation*. Committee on Environmental Impacts Associated with Commercialization of Transgenic Plants. Board on Agriculture and Natural Resources. National Academies Press, Washington, DC.

NRC (National Research Council) (2004) *Biological Confinement of Genetically Engineered Organisms*. National Academies Press, Washington, DC.

Nuffield Council on Bioethics (2004) *The Use of Genetically Modified Crops in Developing Countries*. *A follow-up discussion paper*. Nuffield Council on Bioethics, London.

O'Brochta, D.A., Tonui, W.K., Dass, B. and James, S.A. (2020) Cross-sectional survey of biosafety professionals regarding genetically modified insects. *Applied Biosafety* 25(1), 19–27. doi: 10.1177/1535676019888047

OECD (1986) *Recombinant DNA Safety Considerations. Safety Considerations for Industrial, Agricultural and Environmental Applications of Organisms Derived by Recombinant DNA Techniques* ('The Blue Book'). Organisation for Economic Co-operation and Development, Paris.

OECD (1993) *Safety Considerations for Biotechnology: Scale-up of Crop Plants*. Organisation for Economic Co-operation and Development, Paris.

OECD (2005) *An Introduction to the Biosafety Consensus Documents of OECD's Working Group for Harmonisation in Biotechnology. Series on Harmonisation of Regulatory Oversight in Biotechnology* No. 32. Organisation for Economic Co-operation and Development, Paris.

OGTR (2013) *Risk Analysis Framework 2013, version 4*. Office of the Gene Regulator, Department of Health, Canberra, Australia.

OIE (World Organisation for Animal Health) (2022) Safety, regulatory, and environmental issues related to international trade of insects. *OIE Scientific and Technical Review* Vol. 41 (1) [special edn, Mumford, J. and Quinlan, M.M. (eds)]

OSTP (1986) *Coordinated Framework for Regulation of Biotechnology*. Federal Register. 51:23301–23350. Executive Office of the President, Office of Science and Technology Policy (OSTP), Washington, DC.

Pare Toe, L., Barry, N., Ky, A.D., Kekele, S., Meda, W. *et al.* (2021) Small-scale release of non-gene drive mosquitoes in Burkina Faso: from engagement implementation to assessment, a learning journey. *Malaria Journal* 20(1), 1–18. doi: 10.1186/s12936-021-03929-2

Popovici, J., Moreira, L.A., Poinsignon, A., Iturbe-Ormaetxe, I., McNaughton, D. and O'Neill, S.L. (2010) Assessing key safety concerns of a *Wolbachia*-based strategy to control dengue transmission by *Aedes* mosquitoes. *Memórias do Instituto Oswaldo Cruz* 105(8), 957–964.

Quinlan, M.M. (2014) Assessing risk of transgenic insects. In: Benedict, M.Q. (ed.) *Transgenic Insects: Techniques and Applications*. CAB International, Wallingford, UK, pp. 283–305

Quinlan, M.M. and Enkerlin, W. (2003) The commercialization of SIT. In: *Recent Trends on Sterile Insect Technique and Area-wide Integrated Pest Management – Economic Feasibility, Control Projects, Farmer Organization and Bactrocera dorsalis Complex Control Study*. Research Institute for Subtropics, Naha, Japan, pp. 25–36.

Quinlan, M.M. and Larcher-Carvalho, A. (2007) Tools of the trade: the international business of SIT. In: Vreysen, M.J.B., Robinson, A.S. and Hendrichs, J. (eds) *Area-wide Control of Insect Pests: from Research to Field Implementation*. Springer, Dordrecht, Netherlands, pp. 425–448

Rausand, M. and Haugen, S. (2020) *Risk Assessment: Theory, Methods and Applications*, 2nd edn. John Wiley & Sons, Hoboken, New Jersey.

Raybould, A. (2020) *Hypothesis-Led Ecological Risk Assessment of GM Crops to Support Decision-Making About Product Use*. Science, Technology and Innovation Studies, The University of Edinburgh, Edinburgh, UK.

Regan, H.M., Colyvan, M. and Burgman, M.A. (2002) A taxonomy and treatment of uncertainty for ecology and conservation biology. *Ecological Applications* 12(2), 618–628

Robert, M.A., Okamoto, K., Lloyd, A.L. and Gould, F. (2013) A reduce and replace strategy for suppressing vector-borne diseases: insights from a deterministic model. *PLoS ONE* 8(9), e73233.

Roberts, A., De Andrade, P.P., Okumu, F., Quemada, H., Savadogo, M. *et al.* (2017) Results from the workshop 'Problem formulation for the use of gene drive in mosquitoes'. *American Journal of Tropical Medicine and Hygiene* 96, 530–533. doi: 10.4269/ajtmh.16-0726

Santos, M.R. (2020) Evaluating gene drive approaches for public benefit. In: Chaurasia, A., Hawksworth, D.L. and Pessoa de Miranda, M. (eds) *GMOs: Implications for Biodiversity Conservation and Ecological Processes*. Springer Nature, Cham, Switzerland, pp. 421–437

SCBD (2016) *Guidance on Risk Assessment of Living Modified Organisms and Monitoring in the Context of Risk Assessment*. Secretariat of the Convention on Biological Diversity, Montreal.

Scientists' Working Group on Biosafety (1998) *Manual for Assessing Ecological and Human Health Effects of Genetically Engineered Organisms*. The Edmonds Institute, Edmonds, Washington.

Shelton, A.M., Long, S.J., Walker, A.S., Bolton, M., Collins, H.L. *et al.* (2020) First field release of a genetically engineered, self-limiting agricultural pest insect: evaluating its potential for future crop protection. *Frontiers in Bioengineering and Biotechnology* 7(482)

Simmons, G.S., McKemey, A.R., Morrison, N.I., O'Connell, S., Tabashnik, B.E. *et al.* (2011) Field performance of a genetically engineered strain of pink bollworm. *PLoS ONE* 6(9), e24110. doi: 10.1371/journal.pone.0024110

Spiegelhalter, J.D. and Riesch, H. (2011) Don't know, can't know: embracing deeper uncertainties when analysing risks. *Philosophical Transactions of the Royal Society A* 369, 4730–4750.

Spradling, A.C. and Rubin, G.M. (1982) Transposition of cloned P-elements into *Drosophila* germ line chromosomes. *Science* 218 (4570), 341–347. doi: 10.1126/science.6289435

Stirling, A., Hayes, K.R. and Delborne, J. (2018) Towards inclusive social appraisal: risk, participation and democracy in governance of synthetic biology. *BMC Proceedings* 12(8), 15.

Suter, G.W., Norton, S.B. and Fairbrother, A. (2005) Individuals versus organisms versus populations in the definition of ecological assessment endpoint. *Integrated Environmental Assessment and Management* 1(4), 397–400.

Takken, W., Scott, T.W. and Bogers, R.J. (2002) Ecological aspects for application of genetically modified mosquitoes. In: Takken, W., Scott, T.W and Bogers, R.J. (eds) *Proceedings of the Frontis Workshop on Ecological Challenges Concerning the Use of Genetically Modified Mosquitoes for Disease Control, Wageningen, the Netherlands, 26–29 June 2002*. Frontis – Wageningen International Nucleus for Strategic Expertise, Wageningen University and Research Centre, Wageningen, the Netherlands.

UNCED (United Nations Conference on Environment and Development) (1992) *Rio Declaration on Environment and Development. Annex 1 in: Report of the UNCED, Rio de Janeiro*, Brazil, 3–14 June 1992. A/CONF.151/26 (Vol. I), 12 August 1992.

UNEP (1996) *International Technical Guidelines for Safety in Biotechnology*. United Nations Environment Programme, Nairobi, Kenya.

UNEP (2016) *Guidance on Risk Assessment of Living Modified Organisms and Monitoring in the Context of Risk Assessment*. United Nations Environment Programme, Cancun, Mexico.

UNEP/CBD (United Nations Conference on Environment and Development/Convention on Biological Diversity) (2000) *Cartagena Protocol on Biosafety to the Convention on Biological Diversity*. Secretariat of the Convention on Biological Diversity, Montreal Canada.

UNIDO (1995) *Voluntary Code of Conduct for the Release of Organisms into the Environment*. United Nations Industrial Development Organization, Vienna.

USDA (2016a) *Proposal to permit the field release of genetically engineered diamondback moth in New York*. Biotechnology and Regulatory Services, Animal and Plant Health Inspection Service, US Department of Agriculture. Riverdale, Maryland [see also permit application at 13_297102r_fonsi.pdf (usda.gov)]

USDA (2016b) *Environmental Assessment for Investigational Use of Aedes aegypti OX513A*. Center for Veterinary Medicine. US Food and Drug Administration. Department of Health and Human Services.

USDA-APHIS (2012) *USDA-APHIS-PPQ butterfly environmental release decision chart*. Version 11, April 13, 2012. Available at: www.aphis.usda.gov/plant_health/permits/organism/downloads/decision_chart.pdf (accessed October 2021)

USEPA (1998) *Guidelines for Ecological Risk Assessment*. US Environmental Protection Agency, Washington, DC.

USEPA (2019) *Summary of the Data and Information Related to Vectorial Capacity Presented for the New Product OX5034 (EPA File Symbol: 93167-EUP-E) Containing the Tetracycline-Repressible Transactivator Protein Variant (tTAV-OX5034), a Variant of the Modified Discosoma spp. DsRed2 Protein, and the Genetic Material (Vector pOX5034) Necessary for Their Production in OX5034 Aedes aegypti*. US Environmental Protection Agency, Washington, DC.

USEPA (2020) *Human Health and Environmental Risk Assessment for the New Product OX5034 Containing the Tetracycline-Repressible Transactivator Protein Variant (tTAV-OX5034; New Active Ingredient) Protein a DsRed2 Protein Variant (DsRed2-OX5034; New Inert Ingredient), and the Genetic Material (Vector pOX5034) Necessary for their Production in OX5034 Aedes aegypti*. US Environmental Protection Agency, Washington, DC.

Victoria Government Gazette (2018) *Environment Protection Act 1970. State environment protection policy (Waters)*. Victoria Government Gazette, Melbourne, Australia, S 499.

Waltz, E. (2021) First genetically modified mosquitos released in the United States. *Nature* 593, 175–176.

WHO (1991) *Report of the meeting: Prospects for Malaria Control by Genetic Manipulation of its Vectors*, Tucson, Arizona, 27–31 January 1991. TDR/BCV/MAL-ENT/91. World Health Organization, Geneva, Switzerland.

WHO (2010) *Progress and Prospects for the Use of Genetically Modified Mosquitoes to Inhibit Disease Transmission Report on planning meeting 1: Technical consultation on current status and planning for future development of genetically modified mosquitoes for malaria and dengue control*. World Health Organization, Geneva, Switzerland, 4–6 May 2009.

WHO (2021) *Guidance framework for testing genetically modified mosquitoes*, 2nd edn. World Health Organization on behalf of the Special Programme for Research and Training in Tropical Diseases.

Wolt, J.D. Keese, P., Raybould, A., Fitzpatrick, J.W. Burachik, M. *et al.* (2010) Problem formulation in the environmental risk assessment for genetically modified plants. *Transgenic Research* 19, 425–436.

World Bank (1999) *Environmental Assessment Sourcebook*. Vol. 1. World Bank, Washington, DC.

Index

CABI BIOTECHNOLOGY SERIES

Transgenic Insects 2nd Edition

Techniques and Applications

EDITED BY **MARK Q. BENEDICT** AND **MAXWELL J. SCOTT**

Technology for modifying the genotypes and phenotypes of insects and other arthropods has steadily progressed with the development of more precise and powerful methods, most prominently transgenic modification. For many insect pests, there is now almost unlimited ability to modify phenotypes to benefit human health and agriculture. Precise DNA modifications and gene drive have the power to make wild-type populations less harmful in ways that could never have been performed with previous transgenic approaches. This transition from primarily laboratory science to broader applications for field use has also necessitated greater development of modeling, ethical considerations and regulatory oversight. The 2nd Edition of *Transgenic Insects* contains chapters contributed by experts in the field that cover technologies and applications that are now possible. This edition includes increased attention to associated challenges of risk assessment, regulation, and public engagement. Featuring:

- Up-to-date analysis of molecular techniques, such as gene editing.

- Consideration of public attitudes and regulatory aspects associated with transgenic insects.

- Many examples of the wide range of applications of transgenic insects.

This book will be very valuable to students and researchers in entomology, molecular biology, genetics, public health and agriculture, and will also appeal to practitioners who are implementing the technology, and to regulators, stakeholders and ethicists.

Front cover image: ECFP/DsRed dual-marker transgenic *Anopheles stephensi* mosquito (courtesy of Vanessa Macias and John Cantolina).